PURIFICATION AND IDENTIFICATION OF SULFATED LEWIS X BINDING PROTEINS IN BOAR SPERM

BY

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DISSERTATION

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ABSTRACT

After insemination, sperm can be held in the isthmus region of the oviduct forming a reservoir. There is evidence that formation of the oviduct sperm reservoir is mediated, at least partially, by sperm recognition of carbohydrate structures present on the oviduct epithelium. In pigs, previous studies have proposed that glycans containing mannose termini are the structures recognized by the sperm protein AQN1. However, using a novel screening approach, our group has established that pig sperm bind preferentially to glycans containing the Lewis X trisaccharide (Le^X) or multi-antennary sialylated *N*-acetyllactosamine motifs and much less to motifs with terminal mannose. Based on these findings, the aim of this PhD dissertation was to identify sperm molecule/s involved in sperm binding to the sulfated version of Le^X (sLe^X, a Le^X version that binds sperm with high affinity) that mediate sperm adhesion to the oviduct epithelium. From this work it was possible to detect multiple sLe^X and SiLN putative receptors in epididymal and ejaculated sperm; furthermore, some of these receptors have overlapping characteristics that include comparable molecular weights and requirement of Ca^{2+} for adhesion (Chapter 3). Investigation of sLe^X receptors in ejaculated sperm by affinity chromatography identified the membrane protein AQN1, suggesting that this protein may also recognize glycans with sLe^X termini other than mannose (Chapter 4). A main finding of this work was the establishment of the sperm membrane protein lactadherin as one receptor for the oviduct epithelium; incubation of oviduct cell aggregates with the recombinant protein significantly inhibited sperm adhesion (Chapter 4). Furthermore, additional studies using epididymal sperm, instead of ejaculated sperm, also identified ADAM5 as a receptor for sLe^X (Chapter 5). In summary, the results illustrated in this PhD dissertation prove that distinct proteins are involved in sperm binding to sLe^X motifs and proposes that an adhesion protein complex including lactadherin and ADAM5 binds the oviduct.

DEDICATION

This work is dedicated to my family

ACKNOWLEDGMENTS

I would like to express my deepest gratitude to my PhD program supervisors, Dr David Miller and Dr Rebecca Krisher. They both contributed greatly to my scientific development. Dr Krisher encouraged my passion for oocyte physiology and cell metabolism. Dr Miller has sparked my interest in the glycobiology field and has challenged me with difficult topics that have greatly increased my research skills.

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Contents

List of abbreviations

ACN – acetonitrile

CBD – carbohydrate binding domain

DTT - dithiothreitol

EDTA - ethylenediaminetetraacetic acid

ESI – electrospray ionization

GBP - glycan-binding protein

HBS - hepes buffer saline

kDa - kilodalton

LC- liquid chromatography

Le^X – Lewis X

sLe^X - sulfated Lewis X

LN - *N*-acetyllactosamine

SiLN - sialylated *N*-acetyllactosamine

MS/MS – tandem mass spectrometry

NP-40 - nonylphenyl Polyethylene Glycol

PAGE - polyacrylamide gel electrophoresis

PBS - phosphate buffer saline

RP -HPLC – reversed phase high performance liquid chromatography

SAS – statistical analysis system

SDS- sodium dodecyl sulfate

TBSS - Tris-Buffered sucrose solution

TTBS – Tris/Tween 20 buffer saline

Chapter 1. Introduction

The oviduct is the site of important events during the fertilization process including sperm selection and capacitation, oocyte fertilization and early embryonic development. The oviduct and its fluids provide a suitable environment for fertilization to occur and have profound effect on sperm function. For instance, sperm that have been exposed to uterus and oviduct fluids reach capacitation earlier if compared to sperm capacitated *in vitro*, illustrating the impact of the oviduct milieu on sperm competence (Hunter and Hall 1974). Not only does oviduct secretion influence sperm function, it is recognized that there is a cross-talk between the sperm and oviduct cells that influences oviduct cell gene expression and protein secretion (Fazeli et al. 2004, Georgiou et al. 2005). Therefore, determination of the components regulating the sperm-oviduct cell interaction is of great importance for further understanding of sperm physiology.

During sperm movement in the oviduct, uncapacitated sperm can attach to the isthmic region, establishing a reservoir (Hunter 1981). After capacitation, probably due to alteration of plasma membrane proteins, sperm have reduced capacity to bind oviduct cells (Lefebvre and Suarez 1996). Sperm-oviduct interaction is dynamic and close to ovulation time sperm are released from the reservoir, moving to the ampullatory segment where fertilization occurs. There are three main functions commonly attributed to the oviduct reservoir: 1) **the reservoir prolongs sperm lifespan;** while bound to the oviduct, sperm are resistant to stimulatory components of capacitation and are maintained in an inactive state, 2) **the reservoir reduces polyspermy** and 3) **the reservoir selects a sperm subpopulation.** Therefore, interaction with the oviduct assures the presence of a competent sperm population for fertilization and controls the number of sperm reaching the oocyte.

It is believed that establishment of the oviduct sperm reservoir involves recognition of oviduct glycoproteins by lectin-like receptors present on the sperm plasma membrane. Glycans have several biological functions including involvement in cell-cell adhesion of bacteria to tissues (Hooper and Gordon 2001) or immune responses (Sperandio 2006). In reproductive systems, glycans also play a critical role in cell-cell adhesion; in mice, presence of high mannose *N*-glycans on the germ cell surface facilitates Sertoli–germ cell attachment during spermatogenesis (Huang and Stanley 2010). Current knowledge about the reservoir formation in the pig recognizes mannose as the main carbohydrate termini recognized by the sperm. The sperm counterpart of this interaction is the protein AQN1 that has great affinity to oviduct mannose and galactose carbohydrates.

In an attempt to identify glycans that sperm bind with great affinity, our research group has employed a glycan array approach using 400 candidate glycans. This study has shown that pig sperm preferentially bind to glycans containing Lewis X or multi-antennary sialylated n-acetyllactosamine motifs (SiLN), while there was low binding to mannose motifs. **Based on these findings, I <u>hypothesized</u> that glycan receptors present on the sperm acrosomal region bind to Lewis X motifs to mediate sperm attachment to the oviduct epithelium.** Because Lewis X dimers and trimers reagents were not available, we used sulfated Lewis^{X} (sLe^{X}) reagents which also bind sperm. **My aims were: 1) to detect and characterize sperm receptors for sLe^{X} and SiLN motifs, 2) to identify sLe^{x} receptor/s present on the sperm plasma membrane of ejaculated and epididymal sperm and 3) to elucidate the participation of sperm sLe^{x} receptor/s in oviduct cell adhesion.**

Chapter 2, contains an overall description of current knowledge regarding the oviduct reservoir importance for fertilization and the existing molecules that mediate sperm attachment

to cells. **In Chapter 3**, I characterize receptors to sLe^X, SiLN and *N*-lactosamine motifs based on their location, Ca^{2+} requirement for binding and presence in sperm at different maturation stages, which is part of Aim 1. The results described in this chapter laid the foundation for setting up the protein purification experiments. **In Chapter 4**, I describe the purification of sLe^X-binding proteins using glycan affinity chromatography and further identification of these binding proteins by mass spectrometry analysis, which is part of Aim 2. In addition, in Chapter 4, I also validate the biological importance of the putative sLe^X receptor lactadherin for oviduct binding, which is part of Aim 3. **Chapter 5** focuses in identification of low abundant receptor/s for sLe^X in epididymal sperm and reveals ADAM5 as a putative receptor for sLe^X.

Chapter 2. Literature review -The sperm adhesion machinery

1. Introduction

Glycans refer to a polysaccharide (long carbohydrate unit) or oligosaccharide (short carbohydrate unit; 2-10 monosaccharides) structure. Epithelial cells are surrounded by glycans attached to membrane proteins and lipids, establishing a coating structure known as the glycocalyx (Varki et al. 2009). The glycocalyx is primarily made by monosaccharides (glucose, galactose, mannose, fucose, *N*-acetylglucosamine, *N*-acetylgalactosamine and sialic acid) linked to one another to form a variety of oligosaccharides with specific biological functions (Brandley and Schnaar 1986). There is an increasing interest in studies of saccharide chains and their biological activities. Recognition of glycan structures by membrane proteins mediates cell-cell adhesion and plays an important role in reproductive processes. There are at least two important points of glycan-mediated binding prior to the fertilization process: (1) sperm-oviduct cell binding and (2) sperm-zona pellucida binding. The aim of this chapter is to discuss recent findings that support a novel mechanism of sperm binding to oviduct epithelial cells, focusing on boar sperm. If of interest, another review has discussed the sperm recognition of zona pellucida glycans (Shur 2008).

2. The Oviduct Reservoir

After insemination, sperm pass through the uterine tubal junction within min (Baker and Degen 1972) reaching the oviduct before the uterine immune response occurs (Hunter 1981). The oviduct, also known as the fallopian tubes, is composed of two tubes that connect the uterus to the ovaries. The oviduct is divided into the caudal portion known as isthmus and the cranial portion known as ampulla. During sperm movement in the oviduct, uncapacitated sperm can attach to the isthmic region establishing a reservoir (Hunter 1981). Sperm-oviduct interaction is

dynamic; close to ovulation time sperm are released from the reservoir moving to the ampullatory segment where fertilization occurs.

There are three main functions commonly attributed to the oviduct reservoir:

First, the reservoir prolongs sperm lifespan. While bound to the oviduct, sperm are resistant to capacitating stimulatory components such as bicarbonate (Satake et al. 2006) and are maintained in a state of inactivity characterized by inhibition of capacitation, motility, calcium influx and hyperactivation (Rodriguez-Martinez et al. 2005). Because capacitation destabilizes the plasma membrane and consequently reduces sperm life span (Rodriguez-Martinez 2007), attachment to the oviduct epithelium ensures sperm viability until close to ovulation time. This period may also be considered as a "pre-ovulatory arrest phase" (Hunter 1984).

Second, the reservoir reduces polyspermy. Close to ovulation time, sperm are gradually released from the epithelium controlling the number of sperm reaching the oocyte for fertilization (Mburu et al. 1996). Insemination after ovulation, when oviduct binding is greatly reduced, increases number of accessory sperm bound to oocyte indicating higher number of sperm reaching the oocyte for fertilization (Soede et al. 1995).

Third, the reservoir selects a sperm subpopulation. The oviduct acts as an additional checkpoint for the selection of higher quality sperm. Oviduct cells preferentially bind non-capacitated sperm with an intact acrosome, stable chromatin and reduced protein phosphorylation (Ardon et al. 2008, Fazeli et al. 1999). This sperm population is protected in the oviduct crypts whereas damaged/capacitated sperm are maintained on folds near the central part of the lumen (Mburu et al. 1997). In this region, interaction of capacitated sperm with the oviductal protein *deleted in malignant brain tumor 1* triggers acrosome alterations in a sub

population of prematurely capacitated/damaged sperm reducing the chances of a suboptimal sperm to fertilize an oocyte (Teijeiro and Marini 2012).

3. Glycans: Oviduct Component of Reservoir Formation

In several species, there is evidence suggesting that the oviduct reservoir is mediated by sperm recognition of glycan structures present on the oviduct epithelium (Suarez 2001). The type of oviduct carbohydrate motif recognized by the sperm may be species-specific as sialyl, galactosyl and fucosyl residues competitively inhibit binding of hamster, equine and bull sperm to oviduct epithelium (DeMott et al. 1995, Lefebvre et al. 1997, Sabeur and Ball 2007). Early studies in pigs determined that boar sperm bind oligomannose with high affinity and galactose with low affinity (Wagner et al. 2002). This study was based on a competitive assay using glycans and glycoproteins containing galactose and mannose residues (asialofetuin and ovalbumin, respectively).

The identification of glycans involved in sperm-oviduct binding is often determined by competitive assays based on sperm pre incubation with free candidate sugars or glycoproteins and subsequent determination of binding inhibition to oviduct cells. Even if competitive assays are a valid experimental tool, this approach targets only a small group of candidate carbohydrates. Because of the great diversity of oviduct glycans (Walter and Bavdek 1997), a methodology that tests a broader number of carbohydrates is necessary to identify the authentic sperm binding carbohydrates. Recently, groundbreaking data using a glycan array approach with 377 sugars identified which glycan uncapacitated porcine sperm bind. Noncapacitated sperm bind with great affinity carbohydrate termini that contains a branched sialylated *N*-acetyllactosamine motif (SiLN) or a Lewis X motif (Kadirvel et al. 2012) (Figure 2.1). Binding

was highly specific; for sperm to bind *N*-acetyllactosamine, specific structures are required including a sialic acid linked to the 6 position of galactose and a biantennary conformation. The second motif, Lewis^X, is a trisaccharide containing galactose, fucose and *N*-acetyl glucosamine. Sperm bound preferentially to Lewis^X dimer or trimer, in addition to the sulfated version of Lewis X. Even a small change in fucose position from carbon 3 to 4 forming Lewis A instead of Lewis X oligosaccharide, could be recognized by the sperm and resulted in minimal binding.

Both Lewis X and SiLN motifs are involved in other reproductive events. In humans, the sialylated version of Lewis X is present at the terminus of zona pellucida glycans proteins and is involved in sperm attachment to the zona pellucida during fertilization (Pang et al. 2011). Lewis X carbohydrate has been detected in mouse uterine fluid associated to carrier proteins where it is believed to influence sperm motility and function (Kuo et al. 2009). In pigs, branched sialylated *N*-acetyllactosamine and Lewis X structures are present on the isthmic epithelial cells and it has been suggested that these motifs are part of the sperm recognition mechanism of oviduct cells. Inhibition of oviduct SiLN significantly reduces sperm binding (Kadirvel et al. 2012) supporting the involvement of this carbohydrate as a ligand for sperm surface proteins.

In summary, there is evidence that formation of the sperm reservoir involves recognition of a variety of glycans. In addition to mannose, oviduct Lewis X and multi-antennary sialylated *N*-acetyllactosamine also play an important role in sperm reservoir formation.

4. Sperm Components of Reservoir Formation

Glycans are recognized by lectin-like proteins that have a defined carbohydrate-recognition domain and tend to recognize specific monosaccharide residues by fitting them to a binding pocket (Varki et al. 2009). In the pig, the search for mannose-binding proteins present on the sperm plasma membrane identified members of the spermadhesin family as the proteins

mediating sperm adhesion to oviduct epithelium. Spermadhesins are the major proteins present in boar seminal plasma; members of this family includes AWN, AQN1, AQN3, PSP-I and PSP-II and their glycosylated forms (Topfer-Petersen et al. 1998). Spermadhesins bind to the sperm surface during passage through the epididymis (AWN) or after exposure to accessory gland fluids (AQN1, PSPI and II) coating the membrane covering the acrosomal cap (Dostalova et al. 1994). Spermadhesin AQN1 recognizes glycoproteins with either galactose or oligomannose termini and has been proposed to be the main sperm molecule responsible for oviduct reservoir formation (Ekhlasi-Hundrieser et al. 2005). After capacitation, when sperm adhesion to oviduct cells is greatly reduced, AQN1 is apparently lost from the plasma membrane accounting for sperm release from the oviduct. Antibodies against AQN1 inhibit sperm-oviduct binding, supporting the role of this protein as a glycan-receptor.

Even though early studies have proposed that molecules mediating reservoir formation include carbohydrates with mannose termini and spermadhesin proteins, it is still unclear if other factors are perhaps involved. As previously discussed, branched glycans with SiLN termini play a role in sperm adhesion to oviduct epithelium but the protein recognizing this glycan terminus is still undetermined. Few proteins have been identified as sperm glycan-binding proteins with a recognized physiological function (Table 2.1), and it is not known if these proteins bind Lewis X or SiLN motifs. Recently, a complex containing disintegrin and metalloprotease (ADAM) proteins was proposed as a sperm receptor for zona pellucida *N*-acetyllactosamine but not Lewis X (Mori et al. 2012). It is not known if ADAM proteins also recognize the sialylated form of *N*-acetyllactosamine and furthermore if these proteins have a role in oviduct attachment.

Some Lewis X-binding proteins have been identified in other systems. Selectin proteins are known to recognize a diverse number of Lewis X structures and play a critical role in cancer

metastasis through promotion of cell-cell adhesion (Taylor and Drickamer 2007). A sperm selectin-like protein is thought to recognize fucose residues in sialylated Lewis X motifs present in human zona pellucida, but the identity of this protein still undetermined (Pang et al. 2011).

5. An Expanded Model for Sperm-Oviduct Cell Binding in the Pig

Currently, recognition of mannose and galactose glycans by the sperm is the only reported adhesive mechanism involved in porcine sperm reservoir formation (Topfer-Petersen et al. 2008). Although there is evidence to support the involvement of mannose and galactose residues and their receptor AQN1 in reservoir formation, there is some evidence suggesting that other components also have an important function.

First, based on a glycan array assay, noncapacitated sperm bind with great affinity to Lewis X and SiLN motifs but not mannose.

Second, it is believed that the ability to prolong sperm lifespan is not accomplished by binding to all cells and is restricted to specific cells such as oviduct epithelial cells (Smith and Nothnick 1997). But oligomannose is an intermediary structure in the synthesis of all complex N-glycans (Figure 2.2). Cells that do not completely process oligomannose to complex-type glycans will have glycan structures with terminal mannose. Thus, terminal mannose is found to varying degrees in all cells that display N-glycans and is not restricted to oviduct cells (Varki et al. 2009). Glycans with more unique termini may be required for any oviduct cell-restricted function.

Third, even if fewer epididymal sperm bind to oviduct cells compared to ejaculated sperm (Petrunkina et al. 2001), it is known that epididymal sperm are able to bind oviduct cells. Sperm acquire capacity to bind the oviduct epithelium during passage through the epididymis

(Petrunkina et al. 2001). The mannose and galactose-binding protein AQN1 is produced by seminal vesicles; therefore, it is absent in epididymal sperm (Ekhlasi-Hundrieser et al. 2002). This fact suggests that at least some of the binding components have to be present on epididymal sperm before contact with the accessory gland fluid. Thus, AQN1 may not be the only factor involved in oviduct binding.

6. Acquisition of glycan binding proteins: evidences of a multi-step process

Even if sperm are transcriptionally silent, sperm proteins are under continuous modification during transit through the reproductive tract (Dacheux et al. 1989). During the transport and maturation processes, proteins are removed, added, modified and reorganized on the sperm surface. These protein modifications are critical for sperm function, viability and also binding ability. Caput epididymal sperm bind less to zona pellucida proteins than cauda sperm due to proteins acquired during sperm transit and exposure to epididymal fluid (Burkin and Miller 2000). Based on binding assays using fetuin and asialofetuin glycoproteins, it was determined that distinct proteins mediate epididymal sperm binding to SiLN and Le^X motifs (Mori et al. 2000, Yoshitani et al. 2001).

Even if part of the sperm binding mechanism is present before ejaculation, maximal binding capability to oviduct epithelium is achieved after sperm contact with seminal fluid proteins (Petrunkina et al. 2001). At ejaculation in bull sperm, bovine seminal plasma (BSP) proteins originating from accessory gland fluids interact with choline phospholipids in the plasma membrane to mediate binding to the oviduct epithelium (Gwathmey et al. 2003, Manjunath and Therien 2002). In pigs, spermadhesins mRNA is expressed in the caput and cauda epididymis, but are highly expressed in seminal vesicles accounting for the great amount

of these proteins in ejaculated fluid (Song et al. 2010). Spermadhesin proteins also bind to phospholipids present on the sperm surface (Dostalova et al. 1995). It is necessary to verify the presence and characteristics of glycan binding proteins specific to SiLN and Lewis X on both epididymal and ejaculated sperm plasma membranes to potentially elucidate different mechanism present in each sperm maturation stage.

7. Challenges when studying glycan-binding proteins

The study of glycans and their binding proteins has been a challenge for a variety of reasons, mainly due to heterogeneity and dynamics of glycan structures. Animal cell surfaces are covered by a vast number of different polysaccharides that attach to proteins and lipids present in the plasma membrane forming glycoproteins and glycolipids structures. Some of these topics are discussed here.

A) Glycan multivalency

The density and number of glycan epitopes in a multivalent carbohydrate determines the affinity of the lectin to the glycan ligand (Dam and Brewer 2010b). Interaction between a single monovalent glycan and its binding protein/s is usually very weak. For significant biological overall avidity, it is usually necessary a glycoprotein present multiple copies of the glycan, which is often true of glycoproteins. Binding assays to glycoconjugates may result in erroneous conclusions due to weak binding to monovalent glycan ligands; however, synthesis of multivalent glycan ligands has been used to accurately determine the affinity of multi-valent glycan-protein interactions (Paulson et al. 2006). In addition, mechanisms of enhanced affinity

with density-dependent glycan epitopes may also result in erroneous interpretation (Dam and Brewer 2010a).

B) Glycan dynamic

There are different types of glycans including *N*-glycans that are covalently attached to protein at asparagine residues and O-glycan that are linked via an *N*-acetylgalactosamine moiety to serine or threonine. The formation of a glycan structure occurs in the endoplasmic reticulum and initiates with monosaccharide building blocks that are linked to each other through hydroxyl groups. The glycan chain will be transferred to a protein forming a glycoprotein in the endoplasmic reticulum. All *N*-glycans share a common monosaccharide core composed of Manα1–6(Manα1–3)Manβ1–4GlcNAcβ1–4GlcNAcβ1-Asn-X-Ser/Thr (Molinari 2007). Branches with different monosaccharides attached to the main core account for the diversity of glycan structures (Figure 2.2).

Glycan structures are modified by the presence of glycosyltransferase and glycosidase enzymes that respectively add or remove sugar residues to the main glycan core. Activities of these enzymes have been detected in the oviduct of different species and differ throughout the estrus cycle (Carrasco et al. 2008a, Carrasco et al. 2008b, Tulsiani et al. 1996) suggesting that glycan structures present on the oviduct epithelium may also differ during the estrous cycle. Based on data from our group, SiLN presence on the oviduct epithelium surface does not change during estrous cycle (Kadirvel et al. 2012).

C) Protein dynamic

Epididymal luminal fluid fucosyltransferase and β–D-galactosidase accounts for the modification of sperm surface glycoproteins during sperm maturation (Tulsiani et al. 1998). Therefore, oviductal glycosyltransferases and glycosidases may also modify sperm proteins in the oviduct. Because proteins present on the sperm surface are likely glycosylated proteins, their function may change after exposure to glycosyltransferases or glycosidases in the reproductive tract and consequently alter sperm recognition mechanism. In human sperm, a protein of uterine origin that presents neuraminidase activity induces desialylation of the sperm surface proteins leading to increased sperm binding to zona pellucida (Banerjee and Chowdhury 1997).

D) Recognition of multiple glycans motifs by the same receptor

As previously discussed, different studies have shown that mannose (Ekhlasi-Hundrieser et al. 2005) and SiLN (Kadirvel et al. 2012) are involved in sperm binding to oviduct cells. It is possible that lectin-like proteins may bind several related glycans with different affinity level. The cell adhesion protein *DC-specific intercellular adhesion molecule-3 grabbing nonintegrin* (DC-SIGN) is a C-type lectin known to have dual binding specificities for high mannose oligosaccharides and also Lewis X trisaccharide (Guo et al. 2004). Similarly, AQN1 recognizes both α and β- linked galactose and mannose structures but has much higher affinity for mannose (Topfer-Petersen et al. 1998). In the mouse, the α3-fucosyl residue, present in the Lewis X motif, is required for high affinity binding of mouse sperm to the zona pellucida, in addition to lower affinity binding to a linear β-galactosyl oligosaccharide, illustrating how multiple glycans interact to promote binding (Kerr et al. 2004).

E) Involvement of multiple sperm adhesive molecules

In boar sperm, multiple proteins present on the sperm apical plasma membrane interact with the zona pellucida coordinating the first step in the fertilization process (van Gestel et al. 2007). Therefore, it is also expected that a group of proteins coordinate sperm binding to oviduct cells. For example, the carbohydrate recognition domain of AQN1 is conserved between AQN3 and AWN supporting a coordinated action within members of the spermadhesin family (Ekhlasi-Hundrieser et al. 2008). Current known sperm surface proteins that interact with oviduct cells or zona pellucida are described in Table 2.1. The classical mechanism of cell-cell adhesion involves one glycan structure recognized by one receptor. However, cell-cell adhesion may also involve more elaborate interactions via alternative pathways including a glycan-protein-glycan interaction (Taylor and Drickamer 2007). Since most of the sperm proteins involved in cell-cell adhesion are still undetermined, it is not possible to establish the type of interaction mediating oviduct binding.

8. Conclusions

In the pig, a current proposed model for oviduct reservoir formation includes carbohydrates with terminal mannose and spermadhesin proteins (Ekhlasi-Hundrieser et al. 2005, Wagner et al. 2002). A large scale screen using a glycan array elucidated that noncapacitated boar sperm bind carbohydrate structures containing Lewis X or SiLN motifs. Because novel glycans have been revealed as ligand to sperm proteins, the subsequent step is to determine which proteins function as adhesive molecules to these ligands. Based on these results, it is proposed that a complex adhesive mechanism coordinates sperm binding.

FIGURES AND TABLES

Table 2.1. Characteristic of known glycan receptors that mediate pig sperm binding to zona pellucida and oviduct cells.

Receptor characteristics	AWN[ab]	AQN1[b]	AQN3[bc]	ADAMs[d]
Glycan affinity	Galactose	Galactose Mannose	Galactose	*N*-acetyllactosamine
Present in epididymal sperm	Yes	No	No	Yes
Ca^{2+} required for binding	Yes	Yes	Yes	Unknown
Binding location	Zona pellucida	Oviduct	Zona pellucida	Zona pellucida

[a](Sanz et al. 1992)

[b](Ekhlasi-Hundrieser et al. 2005)

[c](Calvete et al. 1996a)

[d](Mori et al. 2012)

FIGURE 2.1.

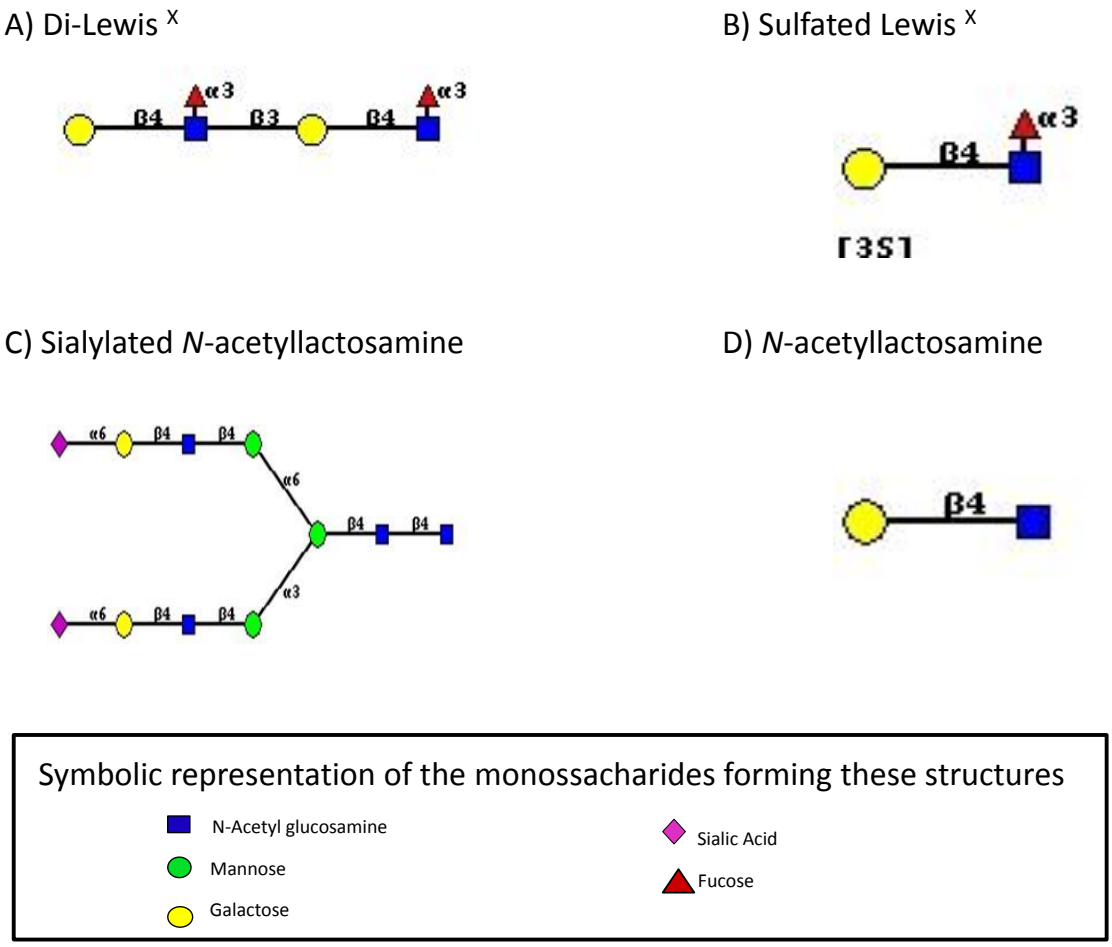

Figure. 2.1. Structures of (A) dimeric Lewis ^{X}, (B) sulfated Lewis ^{X}, (C) biantennary sialylated *N*-acetyllactosamine and (D) *N*-acetyllactosamine motifs.

FIGURE 2.2.

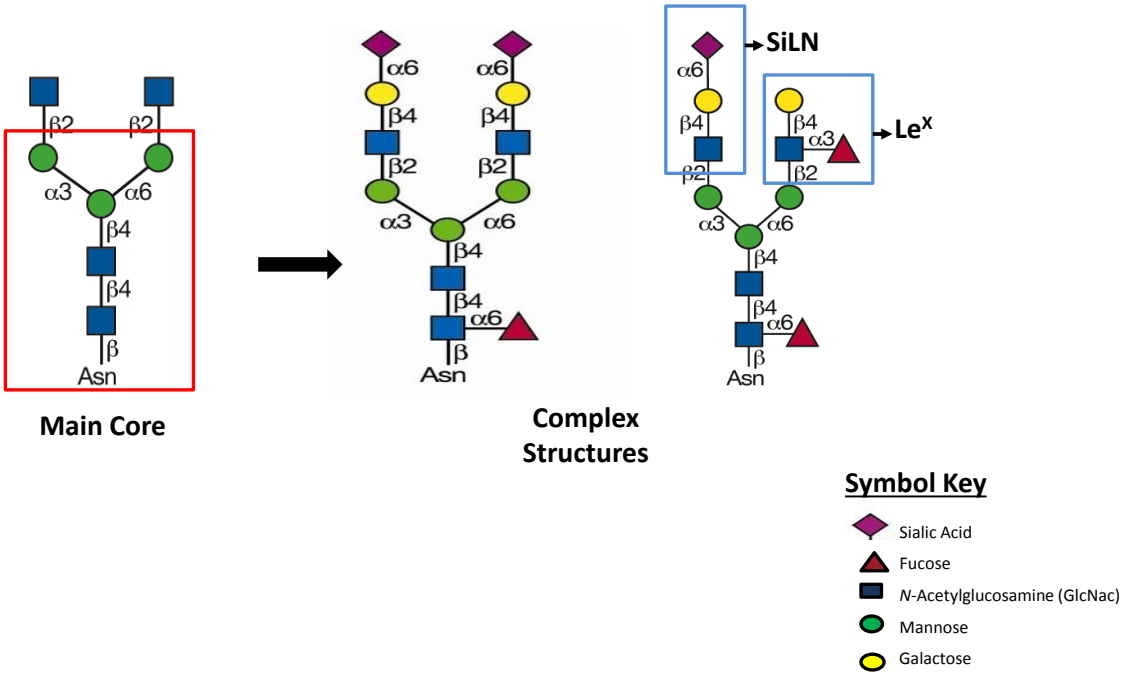

Figure 2.2. Adapted from Varki, RD et al. 2009 (Chapter 8). The *N*-glycan main core consists of $Man_3GlcNAc_2Asn$ (red box). Mannose is an intermediary structure in the synthesis of all complex N-glycans. Further glycan diversification occurs with the addition of monosaccharides to the main core. A single structure can contain hybrid motifs with SiLN and Le^X termini (blue boxes).

Chapter 3. Distribution of sulfated Lewis X and sialylated *N*-acetyllactosamine-binding proteins in ejaculated and epididymal sperm

Abstract

Previously, we demonstrated that porcine sperm bind selectively to Lewis^x (Le^X) and multi-antennary sialylated *N*-acetyllactosamine (SiLN) glycan motifs. Recognition of these motifs mediates sperm binding to oviduct epithelial cells to form a reservoir. Here we characterize proteins that bind to Le^X and SiLN in ejaculated and cauda epididymal sperm. Fluoresceinated 3'-O-sulfated-Le^X (sLe^X) and biantennary SiLN was used to locate the binding proteins on sperm. Binding to *N*-acetyllactosamine (LN) was assessed as a control. Over 60% of ejaculated and epididymal sperm bound to sLe^X and SiLN but only 25% bound to LN. A similar percentage of epididymal and ejaculated sperm bound to both sLe^X and SiLN. Specific binding to sLe^X and SiLN required divalent cations. Fluoresceinated sLe^X bound only to the apical region of the head whereas SiLN bound to a broader area of the head. Further characterization of receptors was accomplished by probing, with specific glycans, sperm proteins that had been separated by SDS-PAGE and transferred to blots. Glycan blotting assays identified several sLe^X-binding bands that migrated as 15 to 250 kDa. Migration of some of the bands that bound SiLN was similar to those that bound sLe^X. A 15 kDa protein was detected in seminal fluid and ejaculated sperm, but absent in epididymal sperm. In summary, we determined that: 1) multiple proteins recognize sLe^X and SiLN motifs, 2) specific binding required divalent cations and 3) Glycan binding proteins were identified before sperm contact with accessory gland fluids. While the identity of these molecules still unknown, this information predicts characteristics of the authentic oviduct sLe^X and SiLN receptors on sperm.

1. Introduction

In several species, after insemination, a small part of the sperm population successfully passes through the utero-tubal junction, reaching the isthmic region of the oviduct where sperm bind to the epithelium to form a reservoir (Hunter 1981). While bound to the oviduct epithelium, sperm are maintained in a protective state characterized by suppression of capacitation and motility, resulting in extended sperm lifespan (Rodriguez-Martinez et al. 2005). In addition to prolonging sperm viability, it has been suggested that oviduct cells recognize and select a more fertile sperm population in mammalian and non-mammalian species (Teijeiro and Marini 2012, Wong et al. 2008). Therefore, interaction with the oviduct increases the likelihood of a competent sperm population for fertilization.

Reservoir formation is mediated by a protein-carbohydrate type of interaction that involves recognition of defined oviduct glycans by glycan-binding proteins located on the sperm membrane (Suarez 2001). The type of oviduct carbohydrate ligands recognized by the sperm may be species-specific as glycans containing sialyl, galactosyl, mannosyl and fucosyl residues competitively inhibit binding of hamster, equine, pig and bull sperm to the oviduct epithelium (DeMott et al. 1995, Lefebvre et al. 1997, Sabeur and Ball 2007, Wagner et al. 2002). While there is general agreement that reservoir formation is mediated by sperm recognition and binding of oviduct carbohydrates, the identity of the molecules involved is still unclear. A recent study used an array of 400 glycans to test which glycans bound boar sperm. Results showed that noncapacitated boar sperm bind glycans with Lewis X trisaccharide motifs (Le^X) and biantennary structures containing a mannose core with 6-sialylated lactosamine at one or more termini (Kadirvel et al. 2012). Because the use of a glycan array allowed a large number of glycan structures to be tested, this study has defined with precision which glycans noncapacitated

sperm bind. Structures containing both glycan motifs have been detected in the female reproductive epithelium and fluid (Kadirvel et al. 2012, Kuo et al. 2009). Blocking sialylated *N*-acetyllactosamine motifs or their putative receptors significantly reduced the ability of sperm to bind oviduct aggregates *in vitro*, demonstrating that this glycan motif is a required ligand for sperm recognition of oviduct cells (Kadirvel et al. 2012).

An essential step to further validate and understand the role of Le^X and sialylated *N*-acetyllactosamine motifs in the formation of the oviduct reservoir is to characterize proteins present on the sperm plasma membrane that recognize and bind these motifs. Glycan-binding proteins are a diverse group of proteins that interact with a carbohydrate ligand to mediate different biological responses such as cell-cell adhesion and cell signaling (Varki 1992). Calcium ions are required for a group of glycan-binding proteins known as C-type lectins to bind carbohydrate structures. Calcium ions interact with hydroxyl groups found on the carbohydrate structure to stabilize binding of the ligand to the protein (Gabius et al. 2011). Calcium is required for bull sperm binding to oviductal cells (Suarez et al. 1998) suggesting that other sperm glycan-binding proteins may also require calcium ion for carbohydrate recognition.

In the pig, AQN1 originating from accessory gland secretions is a known glycan-binding protein involved in sperm adhesion to oviductal cells (Ekhlasi-Hundrieser et al. 2005, Suarez et al. 1998). AQN1 is part of the spermadhesin family of proteins that represents 90% of the total boar seminal plasma proteins and it is peripherally associated with the sperm plasma membrane after ejaculation (Sanz et al. 1993). Binding of sperm AQN1 to oviduct cells is mediated by mannose and galactose residues, not Le^X or sialylated *N*-acetyllactosamine motifs, suggesting the involvement of other proteins in the formation of the sperm reservoir. Although fewer bind, the observation that epididymal boar sperm not exposed to AQN1 can still bind oviduct cells

(Petrunkina et al. 2001) also support the hypothesis that other molecules are involved in sperm binding to the oviduct. Early studies have shown that epididymal sperm contain distinct glycan-binding proteins that mediate recognition of a variety of glycoconjugates ligands as Le^X and sialo-*N*-acetyllactosamine (Yoshitani et al. 2001). Recently, ADAM complex proteins present on epididymal boar sperm surface were identified as putative receptors for *N*-acetyllactosamine (Mori et al. 2012), but it is unknown if this family of proteins would specifically recognize glycans with a biantennary mannose core with 6-sialylated lactosamine.

Our aim was to characterize binding proteins present on the plasma membrane of boar sperm that recognize Le^X and sialylated *N*-acetyllactosamine motifs. Because sperm acquire proteins that enable binding to oviduct cells during movement through the male reproductive tract, we determined if proteins that bound Le^X and sialylated *N*-acetyllactosamine motifs were present on epididymal and ejaculated sperm. Our results lay the foundation for identification of sperm receptors for these ligands that are potential components of the sperm adhesion mechanism.

2. Results

2.1. Localization of sulfated Lewis^X and sialylated N-acetyllactosamine binding proteins on the surface of noncapacitated ejaculated sperm

Although Lewis X dimers and trimers bound sperm (Kadirvel et al. 2012), these reagents were not available and Lewis X multimers were not detected in the oviduct. So as a Lewis X probe, we used sulfated $Lewis^X$ (sLe^X), which is available and binds sperm, along with branched sialylated *N*-acetyllactosamine (SiLN), to localize the putative glycan binding proteins (GBPs) on sperm. First, ejaculated sperm were incubated with fluorescein labeled probes containing

these carbohydrates motifs. We used a non-sialylated form of *N*-acetyllactosamine (LN) as a negative control since sperm require the sialic acid terminus to recognize *N*-acetyllactosamine (Kadirvel et al. 2012). In addition, LN was also used as a negative control for sLe^X because removal of the fucose residue from $Lewis^X$ leaves LN.

There were two distinctive glycan recognition sites: 1) fluorescent signal restricted to the apical head or 2) distributed through the entire head region (Figure 3.1A). Binding to fluoresceinated sLe^X was mostly restricted to the apical head region; more sperm presented this binding pattern when incubated with sLe^X compared to SiLN or LN (67.7, 44.5 and 1.6% of sperm respectively, *P-value* < 0.001, Figure 3.1B). SiLN binding was located in the apical sperm head region but also more broadly distributed over the head, suggesting the presence of multiple binding proteins to this glycan motif.

More sperm bound to fluoresceinated sLe^X and SiLN than LN (61.6, 61.7 and 25.2% of bound sperm respectively, $P<0.0001$, Figure 3.1C). In the presence of the divalent cation chelator EDTA (10 mM), binding to fluoresceinated glycan was significantly reduced for sLe^X and SiLN so that the number of sperm that bound these glycans in the presence of EDTA was equivalent to the number of sperm that bound LN. Thus, specific binding of sLe^X and SiLN required cations such as Ca^{2+} (Figure 3.2).

2.2. Detection of sLe^X and SiLN-binding proteins in whole sperm lysate and ejaculated sperm plasma membrane enriched preparation by glycan blotting.

Glycan-binding proteins were detected in sperm by glycan blotting assay using protein lysates from whole sperm (WS) and plasma membrane-enriched samples (PM). After transfering sperm proteins separated by SDS-PAGE to nitrocellulose, the membrane was probed with

biotinylated glycans. Glycan blot results showed that sperm contains several proteins that recognize sLe^X (Figure 3.3). Enrichment of plasma membrane proteins resulted in the detection of more sLe^X-binding proteins with molecular weight ranging between 15 and 250 kDa. Protein bands of approximately 15 and 37 kDa had stronger signal intensity in PM-enriched samples than WS lysates, suggesting that these GBPs are likely present on the sperm surface (Figure 3.3). A 10x or 100x reduction in the amount of biotinylated sLe^X reduced the number of proteins detected to only four, suggesting that these proteins have greater affinity for sLe^X.

Glycan blotting using biotinylated SiLN also detected several proteins that appeared to have a molecular weight similar to proteins that bound LN. Similar to the sLe^X blot, a protein band of approximately 15 kDa also appears to bind biotinylated SiLN and LN (Figure 3.4A). Proteins that migrated at 37, 50, 57 and 250 kDa were detected in both SiLN and LN glycan blots; however, the signal was faint. To test the hypothesis that similar proteins recognize multiple carbohydrate motifs, we stripped the nitrocellulose membrane after the first incubation with biotinylated SiLN or LN glycans and subsequently re-probed the membrane with biotinylated sLe^X. Re-probing with biotinylated sLe^X revealed the presence of GBPs with molecular weights similar to the first incubation with SiLN and LN (Figure 3.4B).

To determine the origin of the GBPs, we investigated the presence of sLe^X binding proteins in seminal fluid samples. Coomassie staining after a one-dimensional SDS-PAGE gel detected a less complex group of proteins in seminal fluid than in the sperm plasma membrane sample with a major band of 15 kDa. Based on glycan blot results, four GBPs were identified (Figure 3.5). Unexpectedly, glycan blotting assay using seminal fluid protein did not detect proteins that bound SiLN or LN (data not shown).

2.3. Determination of sLe^X and SiLN-binding proteins in epididymal sperm

Because few GBPs were detected in seminal fluid, we decided to assess the presence of GBPs in epididymal (EPI) sperm in an attempt to detect intrinsic sperm proteins that bind these glycan motifs. We investigated the location and MW of GBPs in EPI sperm.

There were no differences in the percentage of EPI and EJA sperm that bound to fluoresceinated sLe^X (73.9% and 74.1% sperm that bound glycan respectively, *P-value* =0.75) and SiLN (74.6% and 70.7% sperm that bound glycan respectively, *P-value*=0.17). Less EPI sperm bound to the control glycan LN than EJA sperm (13.0 vs. 32.1% sperm bound respectively, *P-value* < 0.0001, Figure 3.6).

Inspection of the region of sperm that bound fluoresceinated glycans yielded two binding patterns: 1) fluorescent signal restricted to the apical head region or 2) fluorescent signal covering the sperm entire head sperm (Figure 3.7A). Most EPI sperm presented sLe^X-binding proteins restricted to the apical head region (96.7% of sperm) but fewer sperm bound to this region after ejaculation (71% of sperm, *P-value*< 0.001, Figure 3.7B). The SiLN binding pattern changed more with ejaculation. Fluoresceinated SiLN bound to the apical head region of most (94.3%) of the EPI sperm but only 35.7% of EJA sperm (*P-value* < 0.001, Figure 3.7B). Sperm bound to LN was almost exclusively located present in the entire sperm head for both EPI and EJA sperm (data not shown).

To characterize sperm proteins that bound glycans, sperm proteins were separated by SDS-PAGE, transferred to nitrocellulose and glycan binding proteins detected using biotinylated glycans. After transfer, several proteins from EJA sperm bound to biotinylated sLe^X (Figure 3.8A). The 15 kDa GBP that was present in ejaculated sperm was not detected. Only two major bands with MW of approximately 37 and 40 kDa specifically bound to SiLN and LN (Figure

3.8B). To investigate the origin of GBPs, we analyzed epididymal fluid samples using glycan blotting. No specific signal was detect in epididymal fluid to any of the biotinylated glycans studied (data not shown).

3. Discussion

Recent screening using a glycan array method revealed that noncapacitated porcine sperm bind preferentially to glycans containing the Le^X trisaccharide or multi-antennary SiLN motifs and much less to motifs with terminal mannose or galactose (Kadirvel et al. 2012). Data presented here showing that more sperm bound to fluoresceinated sLe^X and SiLN than the LN motif are consistent with this conclusion. Binding was specific, requiring sialic acid or the fucose monosaccharide added to the LN structure. Our aim in this work was to investigate specific characteristics of proteins that bound sLe^X and SiLN-binding specifically. Establishing these characteristics is important for further identification of the proteins involved in sperm recognition and binding to oviduct epithelial cells.

Based on the location of glycan-binding proteins, we initially hypothesized that sLe^X-binding proteins, mostly restricted to the apical head region, differed from LN-binding proteins that were distributed throughout the entire sperm head. Glycan blotting assays identified multiple sLe^X –binding proteins with molecular weights ranging from 15 to 250 kDa supporting the involvement of different proteins in glycan recognition. It is possible that a single ligand is recognized by a broad class of GBPs because few monosaccharides within the carbohydrate structure are involved in forming a sugar-binding site (Taylor and Drickamer 2009). For instance, bull sperm recognition of oviductal fucose involves the distinctive proteins PDC-109,

BSP-A3 and BSP-30 kDa (Gwathmey et al. 2006). In other cell types, including leukocytes, several proteins also recognize Le^X structures as the C-type selectin family of proteins that bind a diverse number of monosaccharides (Feinberg et al. 2007).

In addition to the paradigm in which multiple proteins recognize the same ligand, there is evidence that a unique GBP may also recognize multiple ligands with different level of affinity. SiLN- binding proteins were localized in both the apical head region and also distributed throughout the sperm head, suggesting that some components involved in SiLN recognition may overlap with sLe^X and LN. Based on glycan blot results, all the proteins that recognized SiLN and LN migrated identically on SDS-PAGE as those that bound sLe^X. Recognition of multiple ligands by the same protein is also not uncommon. It is hypothesized that the diversity of glycan structures available far exceeds the number of potential binding molecules available and each interaction may be associated with distinct protein functions (Taylor and Drickamer 2009). For example, the dendritic cell receptor DC-SIGN has dual-ligand binding properties to high mannose oligosaccharide and Lewis-type structure that are associated with dual-functions in either adhesion or pathogen endocytosis (Guo et al. 2004). In pig sperm, AQN1 protein binds with high affinity to oligomannose residues in addition to low affinity binding to galactose (Wagner et al. 2002). Thus, it is likely that certain GBP that bind sLe^X can recognize SiLN with different degrees of affinity. For adequate detection of SiLN binding by glycan blotting, it was necessary to increase the amount of biotinylated SiLN and membrane exposure time compared to sLe^X; hence, it is possible that if these GBPs are the same, they have greater affinity for sLe^X.

A protein of approximately 15 kDa was consistently detected in plasma membrane and seminal fluid samples in all glycan blots for sLe^X, SiLN and LN. Because this protein was not detected in epididymal sperm by sLe^X blot analysis, it is likely that the 15 kDa protein is attached

to the sperm surface at ejaculation and it is a member of the spermadhesin family of proteins that includes AQN1. It is possible that sperm AQN1 recognizes sLe^X and SiLN in addition to its well-known binding to mannose (Ekhlasi-Hundrieser et al. 2005). Based on sLe^X blot analysis, another 3 proteins with molecular weight of approximately 75, 150 and 250 kDa also potentially originate from seminal fluid. Unfortunately, no specific signal was detected in the SiLN and LN blot analysis of seminal fluid sample; the 15 kDa protein weakly bound to these motifs resulting in a faint signal intensity similar than negative control sample. Further fractionation of the seminal plasma proteins may allow the detection of less abundant SiLN-binding proteins.

A characteristic that is unique to a group of sLe^X and SiLN-binding proteins is the necessity of Ca^{2+} for glycan binding. Incubation of sperm in the presence of EDTA significantly reduced binding to fluoresceinated glycans to levels similar to LN, for which binding was Ca^{2+}-independent. This result suggests that there are two types of molecules involved in binding to sLe^X and SiLN. The first type requires Ca^{2+} to recognize these motifs and therefore may be a C-type receptor. C-type receptors require Ca^{2+} because the protein primary binding site interacts with the monosaccharide through a Ca^{2+} that coordinates the hydroxyl groups of the sugars for binding (Taylor and Drickamer 2009). Earlier study has shown that bull sperm binding to the Lewis A motif is also Ca^{2+} dependent (Suarez et al. 1998). In pigs, sperm recognition of zona pellucida glycans is reported to be Ca^{2+} dependent and mediated by a selectin-type receptor present in the sperm acrosomal membrane (Geng et al. 1997). The second type of sLe^X and SiLN-binding protein recognizes these two ligands in a Ca^{2+}-independent manner. This group of molecules may also recognize LN motifs since this binding was not affected by EDTA. An earlier study determined that Ca^{2+} is not required for epididymal sperm recognition of sialylated and non sialylated lactosamine glycoconjugates and Le^X structures (Mori et al. 2000).

Determination of specific Ca^{2+} requirement for epididymal sperm binding to sLe^X and biantennary SiLN remains to be determined.

During sperm movement in the male and female reproductive tract, the plasma membrane is progressively modified by protein loss, insertion of fluid proteins, glycosylation and changes in lipid composition (Dacheux et al. 1989). Because epididymal sperm are partially competent to bind oviductal cells (Petrunkina et al. 2001), we investigated the presence of GBPs in epididymal sperm and fluid. Competence to bind fluoresceinated sLe^X and SiLN was not different between epididymal and ejaculated sperm. Localization of glycan binding sites demonstrated that sLe^X and SiLN-binding proteins were almost exclusively restricted to the apical head region. The most prominent difference in binding location between epididymal and ejaculated sperm was the alteration in SiLN binding from exclusively restricted to the apical head region in epididymal sperm to the entire head of ejaculated sperm. This observation indicates that some proteins that recognize SiLN are added or relocalized after contact with seminal plasma proteins. Similar to ejaculated sperm, glycan blotting assay detected multiple GBPs to sLe^x in epididymal sperm. The molecular weight of these proteins ranged from 30 to 250 kDa, confirming the absence of the 15 kDa protein.

In summary, a diverse group of proteins recognized sLe^X and few of these proteins also bound SiLN and LN. Because these glycans share some similar structures, a GBP may bind to these motifs but with different affinity. In addition, we determined that there are two distinct groups of proteins that bind sLe^X and SiLN; one that partially requires Ca^{2+} and another that is Ca^{2+}-independent. The Ca^{2+}-independent GBP may also recognize LN. Finally, GBPs to sLe^X and SiLN are already present in epididymal sperm. These results provide a foundation for eventual identification of oviduct glycan receptors on sperm. Because sperm binding to oviduct

cells improves after ejaculation, we suggest that for maximal sperm binding, exposure to accessory gland fluid is necessary.

4. Material and methods

4.1. Material

Chemicals were purchased from Sigma-Aldrich Co. (St. Louis, MO), unless stated otherwise. Carbo-free blocking solution was purchased from Vector Laboratory (Burlingame, CA). SuperSignal West Pico Chemiluminescent Substrate was purchased from Thermo Scientific (Rockford, IL). Precast gels (4-20%) and Precision Plus Protein Standard Kaleidoscope Prestained protein markers were purchased from Bio-RAD (Hercules, CA). Percoll® was purchased from Pharmacia (Piscataway, NJ). Nonylphenyl Polyethylene Glycol (NP-40) was purchased from Calbiochem® EMD Millipore (Billerica, MA). Biotinylated glycans were from Lectinity (Moscow, Russia).

4.2. Ejaculated sperm collection and preparation

Semen from fertile boars was provided by Prairie State Semen Inc. (Champaign, IL). Ejaculates were diluted in Preserv® Xtra extender (Reproquest, Fitchburg, WI) and maintained at 17°C until use. After arrival in the laboratory, semen from 3-4 different boars was pooled and used within 24 h of collection. Before use, semen was washed through a Percoll® cushion as previously described (Kadirvel et al. 2012). Briefly, 3 ml of semen were placed on top of a Percoll® gradient containing 5.4 ml Percoll®, 0.6 ml 10X BSA buffer (1.3 M NaCl, 40 mM KCl, 10 mM $CaCl_2$, 5 mM $MgCl_2$, 140 mM fructose and 0.75M BSA), and 4 ml of dmTALP (2.1 mM $CaCl_2$, 3.1 mM KCl, 1.5 mM $MgCl_2$, 100 mM NaCl, 0.29 mM KH_2PO_4, 0.36% lactic acid,

0.15 mM PVP, 1 mM sodium pyruvate, 35 mM HEPES, pH 7.4). Sperm was centrifuged at 800xg for 10 min. The sperm pellet was resuspended in TALP media and centrifuged again at 600xg for 5 min twice. Only semen with motility > 70% after this procedure was used for experiments.

4.3. Protein extraction from whole sperm lysate

After wash through the Percoll cushion, sperm were incubated with 0.1% NP-40 for 1 h in a cold room (6°C) under gentle rotation. After incubation, lysates were vortexed for 30 sec and subsequently centrifuged for 10min at 10000xg to pellet the insoluble debris. Supernatant was recovered and frozen in a dry ice/alcohol solution. Samples were stored at -20°C until further analysis.

4.4. Seminal plasma collection

Seminal plasma was separated from spermatozoa by centrifugation of boar ejaculate at 1020xg for 30 min at 6°C. The seminal plasma supernatant was recovered and centrifuged one more time at 10000xg for 10 min at 6°C. After the second centrifugation, the supernatant was recovered and frozen in a dry ice/alcohol solution. Samples were stored at -20°C until further analysis.

4.5. Epididymal Sperm Collection

Testes were collected from a local abattoir and were used within 4 hours of slaughter. After arrival in the laboratory, the cauda epididymis was retrograde flushed with PBS (136 mM NaCl, 2.68 mM KCl, 10.4 mM Na_2HPO_4, 1.76 mM KH_2PO_4, pH 7.4) removing sperm. Cauda

epididymal sperm were separated from fluid by centrifugation (800xg for 6 min at 24°C). After centrifugation, cauda epididymal sperm were washed twice with Hepes Buffer Saline (HBS; 126mM NaCl, 5mM KCl, 18.2 mM HEPES, pH 7.4) at 800xg for 6 min, 24°C.

4.6. Plasma membrane isolation from ejaculated and epididymal sperm

An enriched plasma membrane fraction was collected by nitrogen cavitation followed by a sequence of high speed centrifugation steps, as modified from a published protocol (Flesch et al. 1998, Gillis et al. 1978). Washed ejaculated or cauda epididymal sperm were resuspended in Tris-Buffered sucrose solution (TBSS; 40mM Tris Base, 50mM sucrose and SigmaFAST^{TM} Protease Inhibitor Cocktail, pH 7.4). All subsequent media used during this procedure contained protease inhibitor. The sperm suspension ($1x10^9$/ml) was subjected to nitrogen cavitation (10 min, 50 bar) in a cell disruption bomb (Parr Instrument, Moline, IL). The cavitate was slowly released in a 50 ml tube, vortexed for 1 min and centrifuged at 1000xg for 15 min at 6°C. After centrifugation, the supernatant was collected. The pellet was washed with 3 ml of TBSS and centrifuged again at 1000xg for 15 min at 6°C. This procedure was repeated one more time. The supernatants were combined and centrifuged at 5956xg for 20 min at 6°C. The 5956xg supernatant was collected and subsequently centrifuged at 145 250xg for 60 min at 6°C to pellet the membrane proteins. The pellet was washed with HBS and centrifuged again at 145 250xg for 60 min at 6°C. After the last spin, the pellet was collected and solubilized with HBS + 0.1% NP-40 for 30 min on ice. The sample was centrifuged at 10 000xg for 2 min to remove any precipitate. The supernatant was collected and kept at -20°C until further analysis.

4.7. Fluorescent Glycan Binding assay

Localization of glycan binding proteins in live sperm was determined by direct sperm binding to fluoresceinated glycans as previously described with minor modifications (Kadirvel et al. 2012). Sperm concentration was adjusted to 7-8 $x10^7$/ml. An aliquot of 19.5 µl of sperm suspension ($8x10^7$sperm/ml) was incubated with 0.5 µl of fluorescent glycan stock (35 µg/ml final concentration). For a negative control, mTALP media was added instead of glycan. The suspension was incubated for 30 min at 39°C. After incubation with glycans, sperm were immobilized with 0.01% formaldehyde and fluorescence was detected without washing. Sperm immobilization was performed after glycan incubation. After incubation, a small drop (17µl) of sperm suspension was placed on a glass slide and covered with a coverslip. At least 100 sperm were counted per replicate using a 630X oil immersion objective. Positive binding was considered when green fluorescence was detected. All experiments were performed at least 3 times. To determine the influence of Ca^{2+} on sperm binding, sperm were incubated for 30 min with glycans in the absence or presence of the Ca^{2+} chelator EDTA (10mM) in mTBM. After incubation, experiment was carried out as previously described.

4.8. Glycan Blot

After sperm protein determination by BCA protein assay using a BSA standard (Pierce Biotechnology, Rockford, IL), protein (20-15 µg) was solubilized in 4x Laemmli Buffer (40% glycerol, 200mM Tris (pH 8.0), 8% SDS, 400mM dithiothreitol (DTT), 0.1% bromophenol blue) diluted to 1x in sample. Sample was heated at 95°C for 8 min and resolved by gel electrophoresis on 4-20% gradient gels followed by transfer onto a nitrocellulose membrane. The membrane was blocked overnight at 4°C in blocking buffer consisted of 1x Carbo-free blocking buffer diluted in

Tris Buffered Saline and Tween20 (TBST; 50mM Tris Base, 150 mM NaCL, 0.1% Tween 20®, 0.2mM $CaCl_2$ and 0.2mM $MgCl_2$). After the blocking step, the membrane was washed 3 times for 10 min each with TBST and subsequently blotted with 1µg/ml (sLe^X and LN) or 2µg/ml biotinylated glycan (SiLN) diluted in blocking buffer. Additionally, 0.1 and 0.01 µg/ml of sLe^X were tested to help identify proteins with greater affinity for the glycan. After glycan incubation, the membrane was washed 3x for 10 min each with TBST and subsequently incubated for 30 min with Streptavidin Poly-HRP Conjugate (Pierce Biotechnology, Rockford, IL) diluted 1:60,000 in blocking buffer. Proteins that bound biotinylated glycan were visualized after incubation with ECL Western Blotting Substrate (Pierce Biotechnology, Rockford, IL). For a negative control, biotinylated glycan was omitted from the incubation. For the reblotting experiment, the glycan blot probed with biotinylated SiLN or LN glycan membrane was stripped by incubating the membrane in Stripping Buffer (0.2M glycine, 3.46 mM SDS, 1% Tween20, pH 2.2) for 8 min twice. The membrane was subsequently re-blotted with biotinylated sLe^X.

4.9. Statistical analysis

For statistical analysis, the GENMOD procedure of SAS (SAS Institute Inc., Cary, NC, USA) using a probit model with a binary distribution was used to analyze data including: percentage of sperm bound to fluorescent glycans (sLe^X, SiLN and LN), effect of EDTA (present or absent), sperm origin (ejaculated or epididymal) and binding region (apical or entire sperm head). Each sperm was assigned 1 if a fluorescent signal was present or 0 if not present after incubation. For analysis of differences in binding region between glycans, each spermatozoon was assigned 1 if a fluorescent signal was present in the apical head region or 0 if distributed in the entire head. The model included glycan type, EDTA presence or sperm origin depending on

the experiment being analyzed. For analysis of the effect of glycan (sLe^X, SiLN and LN) on sperm binding and binding region, means were separated by using the LSMEANS procedure of SAS when *P-value* <0.05.

Acknowledgement

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FIGURES AND TABLES

Table 3.1. Structures of glycoconjugates used in this study. Glycans were attached to a linear polyacrylamide chain (approximately 30 kDa containing 5% mol biotin and 20% mol saccharide), providing a backbone analogous to a protein in a glycoprotein.

Glycoprotein	Abbreviation	Structure
Sulfated Lewis X	sLe^X	3'-O-SO_3 Galβ4[Fucα3]GlcNac
Biantennary sialylated *N*-acetyllactosamine	SiLN	Neu5Acα2-6Galβ1-4GlcNac β1-2Manα1-3Manβ1-4GlcNac β1-4GlcNac
N-acetyllactosamine	LN	Galβ1-4GlcNac

FIGURE 3.1.

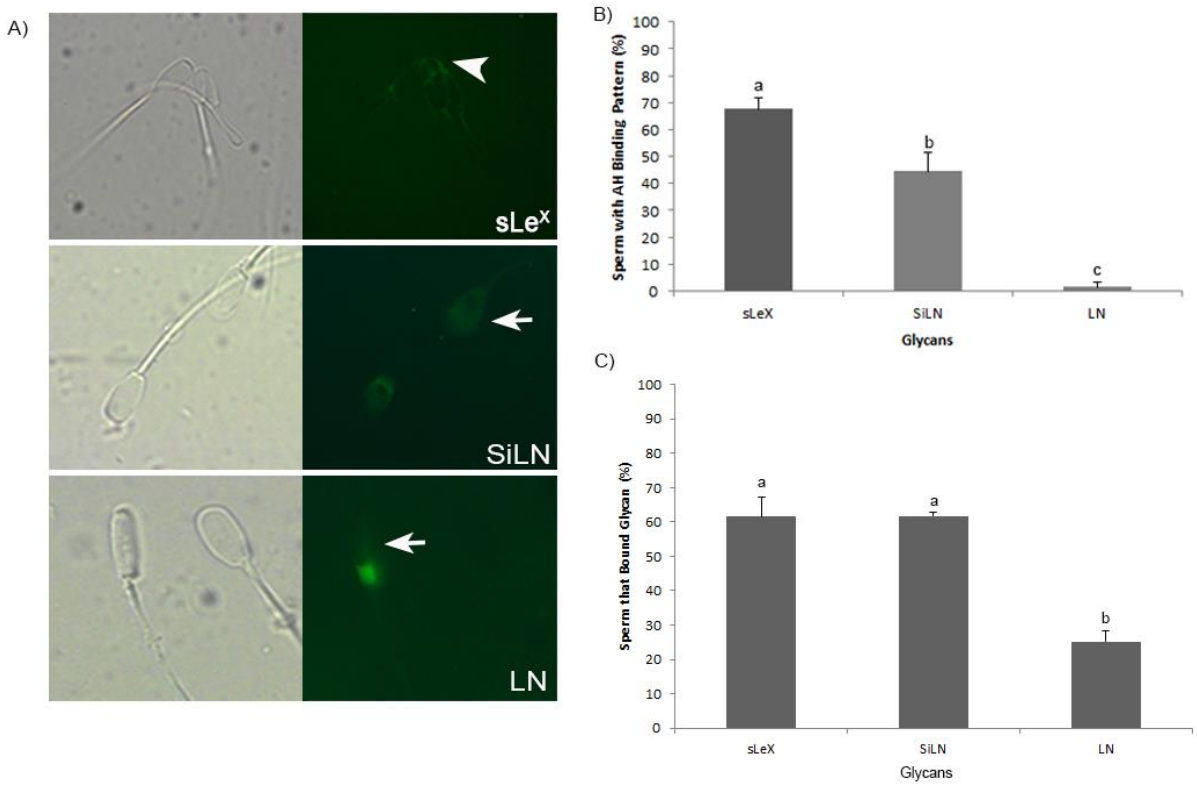

Figure 3.1. Identification of glycan binding sites on ejaculated sperm. A) Localization of sLe^X, SiLN and LN binding sites on sperm by incubation with fluoresceinated glycans (right panel). Two binding patterns (green) were determined; one in which binding was restricted to the apical head (white arrowhead) and the second in which glycan bound the entire head (white arrow). The corresponding brightfield images are shown on the left panel. B) Of those sperm that bound fluoresceinated glycans, most binding to sLe^X was restricted to the apical head (AH) whereas binding to LN was mostly distributed throughout the sperm head. C) The percentage of fluorescent sperm was compared between glycans. More sperm bound to either SiLN or sLe^X than LN. Superscripts with different letters differ ($P< 0.001$). Bars represent the average of 3-4 replicates and SEM.

FIGURE 3.2.

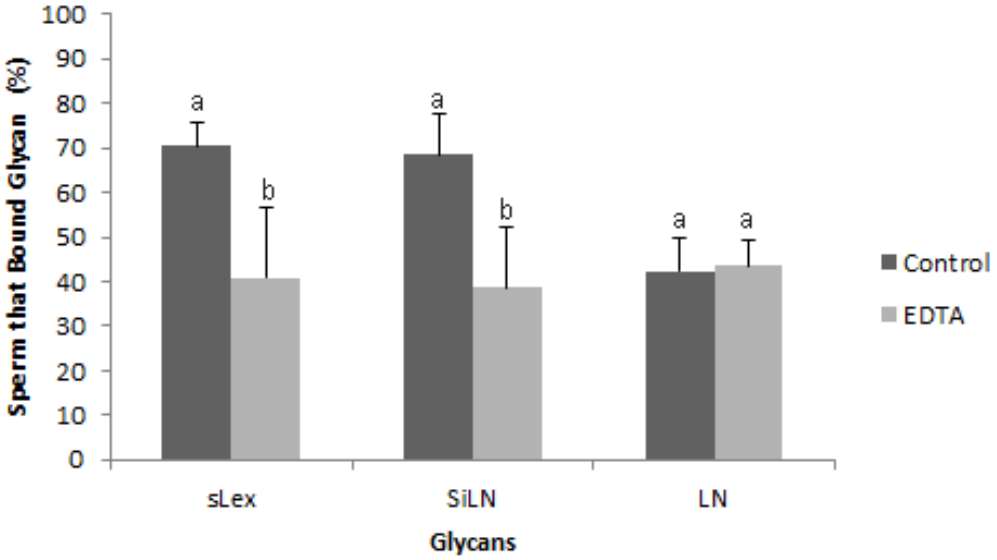

Figure 3.2. Calcium was required for sperm binding to sLe^X and SiLN. Sperm were incubated with fluoresceinated sLe^X, SiLN and LN in the absence (Control) or presence of 10 mM EDTA (EDTA). After 30 min incubation, the number of sperm with a fluorescent signal was determined. Superscripts with different letters differ within each glycan group ($P< 0.001$). Bars represent the average of 3 replicates and SEM.

FIGURE 3.3.

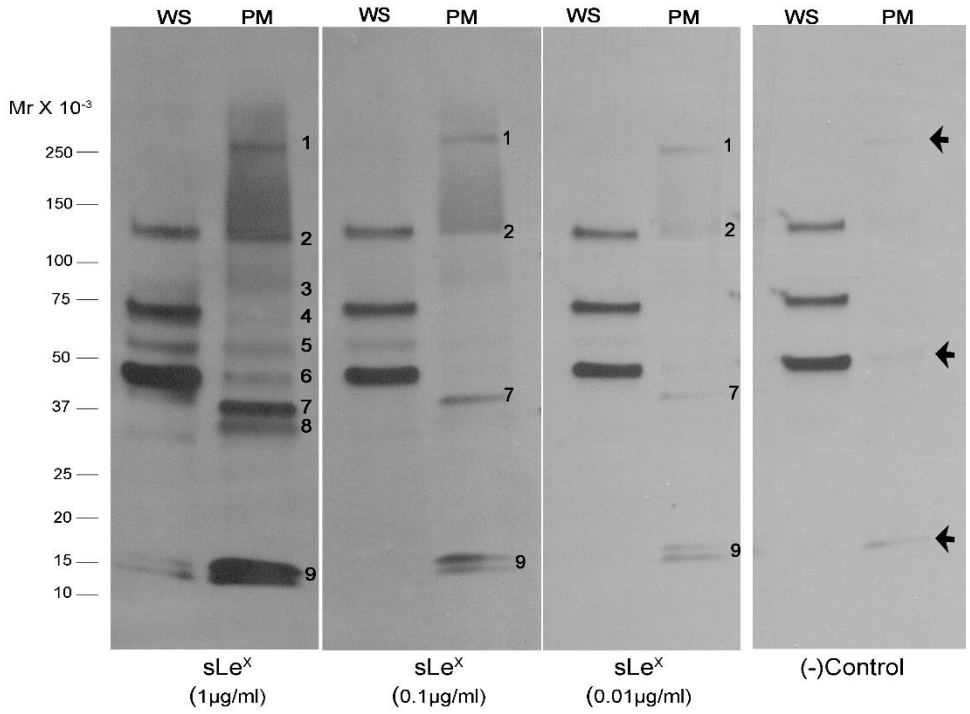

Figure 3.3. Identification of sLe^X-binding proteins in whole sperm (WS) and plasma membrane lysates (PM) of ejaculated sperm. Protein (15µg) was separated by 4-20% SDS-PAGE and transferred to membrane. Membrane was probed with varying concentrations of biotinylated sLe^X (1, 0.1 or 0.01 µg/ml). Proteins with high signal intensity are numbered for PM lane. As a negative control (- Control), the biotinylated glycan was omitted. Nonspecific bands of 50, 75 and 140 kDa were detected for all blots using WS lysate and also a faint intensity signal is present for PM (black arrows). The position of the protein standards is shown on the left.

FIGURE 3.4.

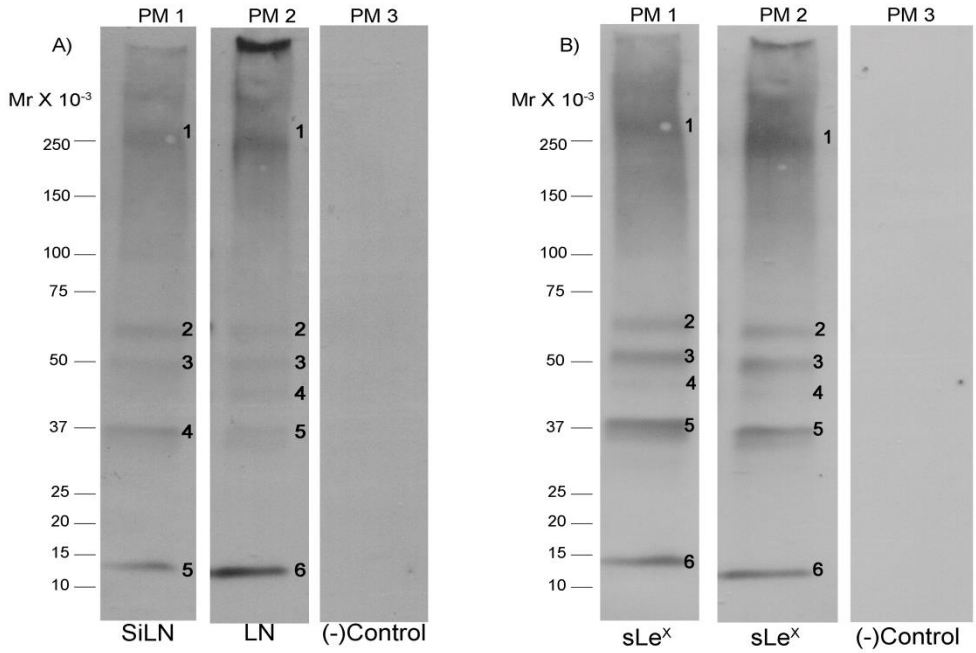

Figure 3.4. Identification of SiLN-binding proteins in plasma membranes of ejaculated sperm. A) Protein (15µg) was separated by one-dimensional 4-20% SDS-PAGE and probed with biotinylated SiLN (PM1) or LN (PM2). Proteins with high signal intensity are numbered for PM1 and PM2. As a negative control (- Control) the biotinylated glycan was omitted (PM3). Membranes were exposured for 1 hour to detect the chemiluminescent signal. B) After the first incubation with biotinylated SiLN (PM1) or LN (PM2), membranes were stripped and reblotted with biotinylated sLe^X. Membranes were exposed for 4 min to detect chemiluminescent signal. Proteins with high signal intensity are numbered for PM1 and PM2 and are similar to the protein signals present in the first blot. The position of the standard marker proteins are shown on the left for both blots.

FIGURE 3.5.

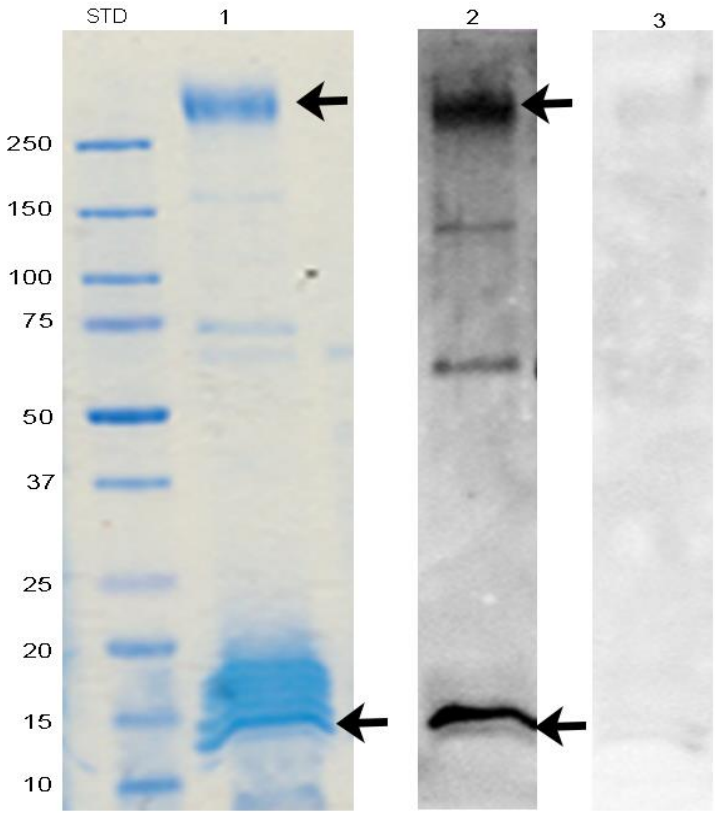

Figure 3.5. Identification of sLe^X-binding proteins in seminal fluid. Proteins (20 µg) from seminal fluid were separated by 4-20% one-dimensional SDS-PAGE and subsequently stained by Coomassie Blue (Lane 1) or blotted with biotinylated sLe^X (Lane 2). Black arrows indicate protein bands with similar molecular weight between Lane 1 and 2. In the negative control (Lane 3), the biotinylated sLe^X was omitted. Faint nonspecific staining of the 15 kDa protein appears in Lane 3. The position of the standard marker proteins are shown on the left (STD).

FIGURE 3.6.

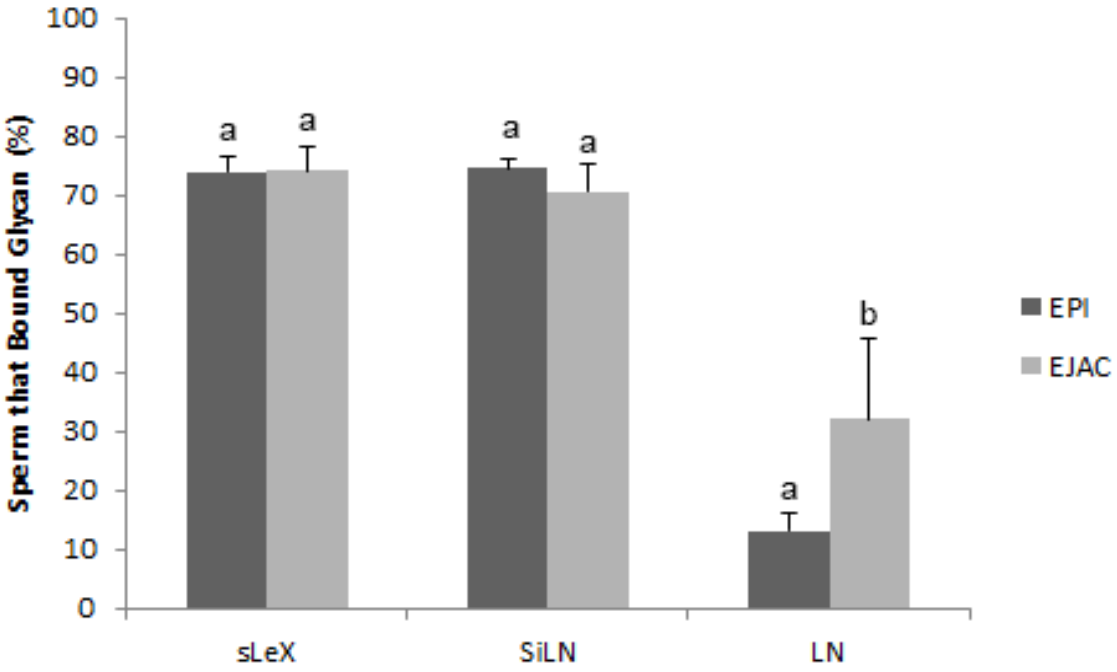

Figure 3.6. Comparison of binding competence to fluoresceinated glyconjugates between epididymal and ejaculated sperm. Sperm were incubated with fluoresceinated sLe^X, SiLN or LN. The number of sperm with a fluorescent signal was determined and compared between epididymal and ejaculated sperm. Superscripts with different letters differ ($P< 0.001$) within each glycan type. Bars represent the average of 3 replicates and SEM.

FIGURE 3.7.

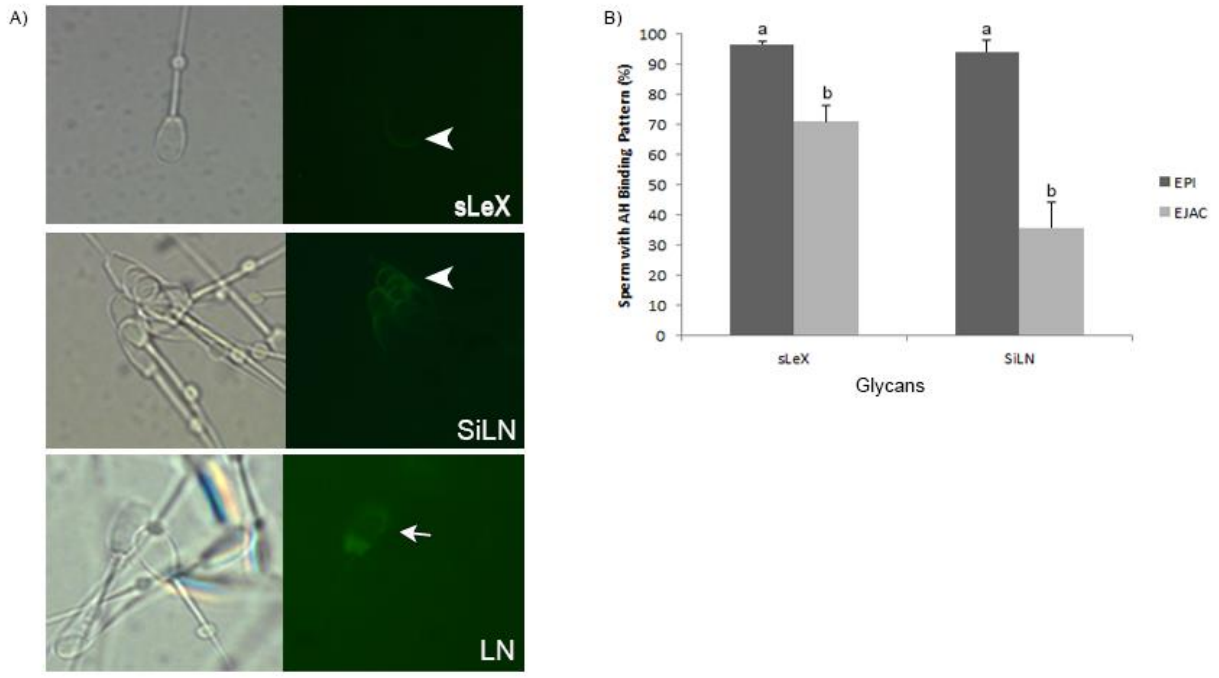

Figure 3.7. Identification of glycan binding sites in epididymal sperm. A) Sperm were incubated with fluoresceinated sLe^X, SiLN and LN. After incubation, binding was detected by fluorescence microscopy and fluorescent sperm were classified as having glycan bound to the apical region (white arrowhead) or the entire head (white arrow). The corresponding brightfield images are shown (left panel). B) Quantification of the number of sperm presenting a glycan binding pattern restricted to the apical head (AH) region. Most epididymal sperm presented sLe^X and SiLN bound to the apical head. After ejaculation the percentage of sperm presenting this binding pattern was reduced. Superscripts with different letters differ within a glycan type ($P< 0.001$). Bars represent the average of 3 replicates and SEM.

FIGURE 3.8.

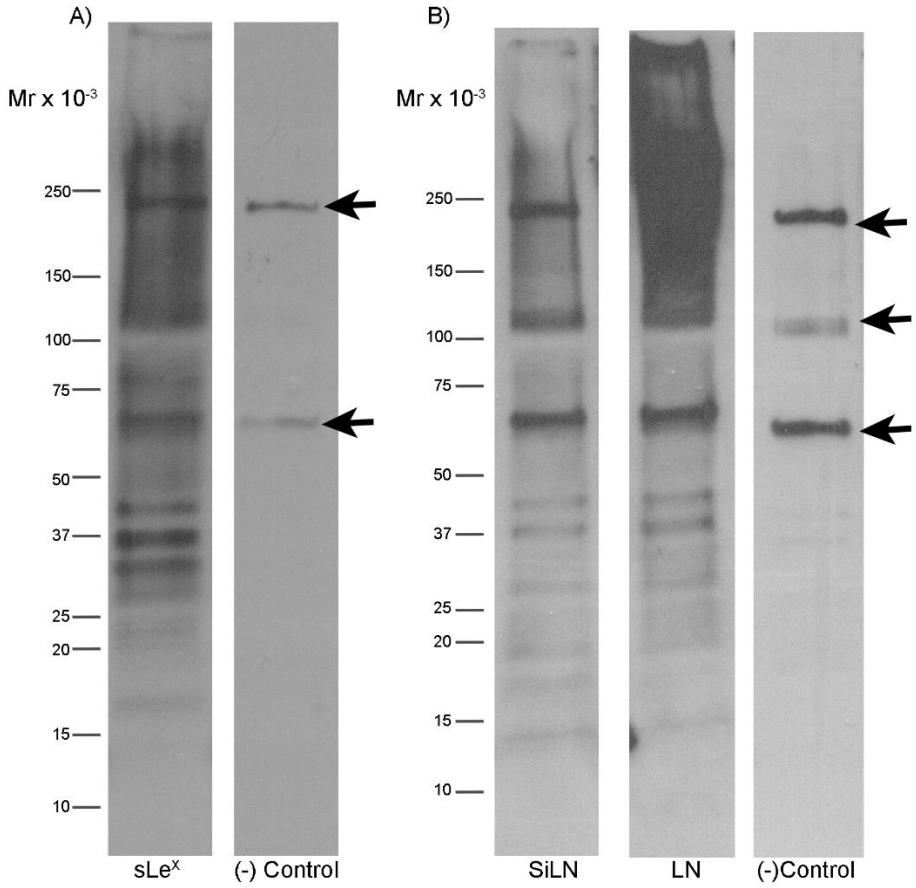

Figure 3.8. Identification of glycan binding proteins in plasma membrane lysates of epididymal sperm. A) After protein (15µg) separation by SDS-PAGE and transfer, protein was blotted with sLe^X (1 µg/ml). In the negative control (-Control), the biotinylated sLe^X was omitted. Nonspecific staining of two protein bands was observed (black arrow). B) Blotting with SiLN and LN glycans (1 µg/ml). Nonspecific staining of three protein bands was observed (black arrow). Representative blots are shown. The position of the protein standards is shown on the left.

Chapter 4. Identification of multiple Lewis X-binding proteins in ejaculated boar sperm

Abstract

Attachment to oviduct glycan motifs including Lewis X structures (Le^X) is important for sperm retention to the oviduct epithelium and formation of a reservoir. The objective of this study was to purify relevant sLe^X-binding proteins that are involved in adhesion to the oviduct. Boar sperm lysate were submitted to a glycan affinity column followed by 1-D SDS PAGE and Coomassie staining. Five protein bands were identified (P14, P27, P44, P56 and P100) and submitted to LC-MS/MS. P14 included several proteins that belong to the spermadhesin family (AWN, AQN1, AQN3, AWN and seminal plasma protein pB1). P27 was identified as ESBP1, known to form a complex with spermadhesin AQN1. Proteins in P44 included two isoforms of lactadherin, (MFGE8 and B2CZF8). P56 and P100 were identified as cytoplasmic proteins likely isolated due to their association with other molecules. Using glycan blotting assays, lactadherin was confirmed as a sLe^X-binding protein. Additionally, competitive assays using recombinant mouse lactadherin established that this protein mediates sperm adhesion to oviduct cells. In summary, these findings reveal that multiple proteins may coordinate sperm binding to the sLe^X motif. In addition, lactadherin was established as one the molecules mediating sperm-oviduct binding. These findings demonstrate that sperm lactadherins are receptors that bind oviduct glycans that contribute to sperm adhesion to oviduct epithelial cells.

1. Introduction

After mating or insemination, mammalian sperm pass through the utero-tubal junction and bind to the oviduct epithelium forming a reservoir (Hunter 1984, Smith and Yanagimachi 1991, Suarez 1987). Binding to the oviduct epithelium maintains sperm in an uncapacitated state characterized by inhibition of motility and hyperactivation, thereby extending sperm lifespan (Rodriguez-Martinez et al. 2005). Sperm are maintained bound to the epithelium until close to the time of ovulation when sperm are gradually released and move toward the oocyte (Mburu et al. 1996). By binding sperm, the oviduct increases the odds that oocytes will be exposed to competent sperm for fertilization (Rodriguez-Martinez et al. 2005). Formation of the oviduct reservoir is mediated by sperm recognition of oviduct carbohydrate structures; the type of oviduct carbohydrate motif recognized by the sperm appears to be species-restricted as sialyl, galactosyl, mannosyl and fucosyl residues competitively inhibit binding of hamster, equine, boar and bull sperm to oviduct cells (DeMott et al. 1995, Lefebvre et al. 1997, Sabeur and Ball 2007, Wagner et al. 2002). Although these monosaccharides inhibit sperm binding to oviduct cells competitively, the authentic oviduct glycan that binds sperm is unknown.

A recent study has demonstrated that boar sperm recognize, with high affinity, carbohydrate structures containing Lewis X and branched sialylated *N*-acetyllactosamine motifs, and that recognition of these motifs mediates boar sperm binding to the oviduct cell aggregates (Kadirvel et al. 2012). Lewis X is a trisaccharide formed by the monosaccharides *N*-acetyl glucosamine, galactose and fucose. Sperm adhesion to Lewis X or the sialylated version of Lewis X is proposed to mediate sperm binding to the human oocyte zona pellucida (Kerr et al. 2004, Pang et al. 2011). Although receptors for this motif have not been identified in boar sperm, binding assays to fluorescein labeled glycans have shown that sperm recognize Lewis X motifs and that

receptors are located overlying the anterior acrosomal region, the region of sperm that binds oviduct cells (Silva et al. 2013).

To assess the biological importance of sperm binding to glycans containing Lewis X termini, it is essential to identify Lewis X receptors. In addition to the initial screen for potential receptors, this manuscript reports, for the first time, that the protein lactadherin binds glycans and is necessary for normal sperm binding to oviduct epithelial cells.

2. Material and methods

In this study, a multiple step strategy was used to purify and identify glycan binding proteins with the use of a glycan affinity column, one-dimensional SDS-PAGE, glycan blotting and LC-MS/MS. The experimental design is outlined in Figure 4.1.

2.1. Materials

Chemicals were from Sigma-Aldrich Co. (St. Louis, MO), unless stated otherwise. Carbo-free blocking solution was from Vector Laboratory (Burlingame, CA). SuperSignal West Pico Chemiluminescent Substrate was from Thermo Scientific (Rockford, IL). Precast gel (4-20%) and Precision Plus Protein Standard Kaleidoscope Pre stained protein markers were from Bio-Rad (Hercules, CA). Percoll® was from Pharmacia (Piscataway, NJ). NP-40 was from Calbiochem® EMD Millipore (Billerica, MA). SimplyBlue™ SafeStain was from Life Technologies (Carlsbad, CA). The BCA assay kit was from Pierce Biotechnology (Rockford, IL). Biotinylated glycans were from Lectinity (Moscow, Russia).

2.2. Collection of ejaculated and epididymal sperm lysate

Pooled semen from fertile boars was provided by Prairie State Semen Inc. (Champaign, IL) and washed through a Percoll® cushion as previously described (Kadirvel et al. 2012). Cauda epididymal sperm was flushed retrograde with PBS (136 mM NaCl, 2.68 mM KCl, 10.4 mM Na_2HPO_4, 1.76 mM KH_2PO_4, pH 7.4) and further separated from epididymal fluid by centrifugation at 800xg for 6 min (24°C). Washed ejaculated and epididymal sperm were extracted with 0.1% NP-40 diluted in Hepes Buffer Saline (HBS; 126mM NaCl, 5mM KCl, 18.2 mM HEPES, pH 7.4) for 1h in a cold room (6°C) under gentle rotation. After incubation with NP-40, sperm suspensions were vortexed for 30 seconds and subsequently centrifuged at 10000xg for 10 min to pellet the insoluble debris. Supernatant was recovered and frozen in dry ice. Samples were stored at -20°C for further use.

2.3. Plasma membrane isolation from ejaculated and epididymal sperm

An enriched plasma membrane fraction was collected by nitrogen cavitation followed by a sequence of high speed centrifugation steps, as modified from a published protocol (Flesch et al. 1998, Gillis et al. 1978). Washed ejaculated or cauda epididymal sperm were resuspended in Tris-Buffered sucrose solution (TBSS; 40 mM Tris Base, 50 mM sucrose and SigmaFAST^{TM} Protease Inhibitor Cocktail, pH 7.4). All subsequent media used during this procedure contained this protease inhibitor cocktail. The sperm suspension ($1x10^9$/ml) was subjected to nitrogen cavitation (10 min, 50 bar) in a cell disruption bomb (Parr Instrument, Moline, IL). The suspension was slowly released from the Parr bomb into a 50 ml tube, vortexed for 1 min and centrifuged at 1000xg for 15 min at 6°C. After centrifugation, the supernatant was collected. The pellet was washed with 3 ml of TBSS and centrifuged again at 1000xg for 15 min

at 6°C. This procedure was repeated one more time. The supernatants were combined and centrifuged at 5956xg for 20 min at 6°C. The 5956xg supernatant was collected and subsequently centrifuged at 145,250xg for 60 min at 6°C to precipitate the membrane proteins. The pellet was washed with HBS and centrifuged again at 145,250xg for 60 min at 6°C. After the last centrifugation, the pellet was collected and solubilized with HBS + 0.1% NP-40 for 30 min in ice. The sample was centrifuged at 10,000xg for 2 min to remove any precipitate. The supernatant was collected and kept in -20°C until further analysis.

2.4. Glycan Blot

Protein samples (15 µg) and recombinant lactadherin were diluted in 4x LSB (10% glycerol, 50 mM Tris Base, 2% sodium dodecyl sulfate, 100 mM DTT, 0.025% Bromophenol Blue) and heated at 100°C for 7 min. Protein samples were resolved by SDS-PAGE performed on 4-20% gradient gels followed by transfer onto a nitrocellulose membrane. The membrane was blocked overnight at 4°C in blocking buffer consisting of 1x Carbo-free blocking buffer diluted in Tris Buffered Saline and Tween20 (TBST; 50mM Tris Base, 150 mM NaCL, 0.1% Tween 20®, 0.2mM $CaCl_2$ and 0.2mM $MgCl_2$). After the blocking step, the membrane was rinsed with TBST (2x, 10 min) and subsequently blotted with 0.5µg/ml biotinylated sLe^X. After glycan incubation and rinsing, bound sLe^X was visualized by incubation with Streptavidin Poly-HRP Conjugate (Pierce Biotechnology, Rockford, IL) diluted 1:60,000 in blocking buffer for 30 min and developed using ECL Western Blotting Substrate (Pierce Biotechnology, Rockford, IL). For negative control, a membrane was incubated in buffer without biotinylated glycan.

2.5. Western blot

After protein preparation and transfer as described for glycan blotting, the membrane was blocked in TBST containing 5% milk for 1 hour at room temperature. After rinsing, the membrane was incubated with polyclonal mouse lactadherin antibody (R & D Systems, AF2805) in TBST containing 2% milk for 1 hour. The bound antibody was visualized by incubation with goat IgG horseradish peroxidase-conjugated antibody (R & D Systems, HAF019) for 30 min and developed using ECL Western Blotting Substrate (Pierce Biotechnology, Rockford, IL). Rinsing steps were performed with TBST. Recombinant lactadherin protein (R & D Systems, 2805-MF/CF) was used as a positive control. The antibody recognized the recombinant protein in reduced and non-reduced conditions (Figure 4.7) so results with sperm proteins are shown under reducing conditions.

2.6. Purification of sulfated Lewis X-binding proteins from ejaculated sperm

Protein extracted (18mg) from whole sperm lysate was incubated overnight with Sepharose beads covalently bound to sulfated Lewis X diluted in 10 ml of Buffer A (20mM Tris-HCl, 0.1 M NaCl, 2mM $CaCl_2$, 2mM $MgCl_2$, protease inhibitor cocktail, pH 7.8). The sperm and Sepharose bead suspension was incubated with gentle agitation at 4°C. After overnight incubation, the suspension was centrifuged at 600 rpm for 5 min to pellet the beads. The collected supernatant was considered the first flow-through fraction (F1) and it was kept for further analysis. The Sepharose beads were resuspended in 10ml of Buffer A and re-centrifuged. After supernatant collection, beads were resuspended in 2 ml of Buffer A and mixture transferred to a disposable minicolumn and sequential flow-through fractions were collected.

Proteins were eluted from the column using 2 ml Elution buffer (20mM Tris-HCl, 0.5M NaCl, 10mM EDTA, 0.1%NP-40, protease inhibitor cocktail, pH7.8). The use of NaCl in concentrations between 0.1-1M in elution buffer improves the recovery of glycoproteins from lectin affinity chromatography through disruption of protein-glycan interaction (Lotan et al. 1977). In addition, EDTA was added in the elution buffer based on previous data demonstrating that protein-Lewis X binding at least partially requires Ca^{2+} (Silva et al. 2013). The elution procedure was repeated 6 times. All 6 eluted fractions recovered from the affinity column were concentrated to a volume of approximately 150-200 µl by a filter device (Amicon Ultra4, 10K, Millipore, Billerica, MA, USA) and further assayed for protein concentration using the BCA assay and a BSA standard. The presence of glycan binding proteins was confirmed by glycan blot assays. A second technical replication of the glycan affinity purification process was completed to demonstrate reproducibility. Affinity chromatography fractions were resolved by one-dimensional SDS-PAGE using a 4-20% Tris-Glycine gel. The gel was stained with SimplyBlue™ SafeStain, and five protein bands of interest observed in the first eluted fraction were excised from the gel.

2.7. In-gel Tryptic digestion

Excised bands were washed and destained, and ProteaseMax™ surfactant-assisted in-gel protein digestion (Promega, Madison, WI) was carried out according to manufacturer's recommended protocol. Gel slices were cut into 1 mm^3 pieces and washed once with water and twice with 50% MeOH/50 mM NH_4HCO_3. The pieces were dehydrated with 50% ACN/50 mM NH_4HCO_3 and 100% ACN washes followed by drying *in vacuo* using a Thermo Scientific Savant SpeedVac concentrator (SVC100, Waltham, MA). Proteins were reduced by incubation

in a solution of 25 mM DTT in 50 mM NH_4HCO_3 at 56 °C for 20 min. The solution was discarded and proteins were alkylated by incubation in a solution of 55 mM IAA in 50 mM NH_4HCO_3 at room temperature for 20 min in the dark. The pieces were washed twice with water, dehydrated as previously described, and dried *in vacuo*. Digestion was performed by rehydrating the pieces in a solution of 12 ng/µL sequencing-grade trypsin and 0.01% ProteaseMax surfactant in 50 mM NH_4HCO_3 for 10 min at room temperature, overlaying with a solution of 0.01% ProteaseMax surfactant in 50 mM NH_4HCO_3, and incubating at 37 °C for 2 hr. The samples were then centrifuged briefly and the solution containing the extracted peptides was transferred to a fresh sample tube. Formic acid was added to a final concentration of 0.5% (pH ≤ 3), and the peptides were desalted using $ZipTip_{C18}$ (Millipore, Billerica, MA) solid phase extraction pipette tips. Peptides were dried *in vacuo* and reconstituted in 20 µL 0.1% formic acid for nano LC-ESI-MS/MS analysis.

2.8. Nano LC-ESI-MS/MS analysis

All experiments were performed on a system consisting of a Waters NanoAcquity UPLC (Milford, MA) interfaced with a Thermo Scientific Q-Exactive mass spectrometer (Bremen, Germany) through a nanoelectrospray ion source. Mobile phase A consisted of 0.1% formic acid in H_2O, and mobile phase B consisted of 0.1% formic acid in ACN. Four microliters of each sample were injected onto a Waters Symmetry C18 5 µm, 180 µm × 20 mm trap column at a flow rate of 5 µL/min for 5 min at 99% A 1% B. Separation was performed on a Waters BEH130 C18 1.7 µm, 75 µm × 150 mm analytical column using a linear gradient of 5 to 35% mobile phase B at 300 nL/min over 90 min. The Q-Exactive was operated in data dependent mode to automatically switch between survey scan MS and MS/MS acquisition. Survey scan spectra were

acquired in the orbitrap mass analyzer using a mass range of 200-2000 *m/z* at a resolving power of 70,000 FWHM (*m/z* 200) with an AGC target of 1e6. The top ten precursor ions were sequentially selected for HCD fragmentation and MS/MS analysis in the orbitrap at a resolving power of 17,500 with an AGC target of 1e5. MS/MS scans employed an isolation window of 3 Da and a normalized collision energy of 30. Precursors were subject to dynamic exclusion.

2.9. Data Processing

Resulting data were processed using Proteome Discover 1.4 software suite (Thermo Fisher Scientific, San Jose, CA). Mascot search algorithm (Matrix Science) was performed against the UniProt *Sus Scrofa* complete database (October 2012) to identify proteins. Reported peptides were filtered to a false discovery rate of 1%. Reported proteins were filtered to require a Mascot score greater than 50 and at least two unique peptides used for identification. Contaminant proteins such as keratins were not included in the reported identifications.

2.10. Oviduct binding assay

Details of the sperm-oviduct binding assay are described elsewhere (Kadirvel et al. 2012). This assay was used with minor modifications. Briefly, 20 oviduct cells aggregates were incubated with increasing concentrations of recombinant mouse lactadherin (R & D Systems) for 30 min. Sperm (final concentration $1x10^6$ sperm/ml) were added to aggregates and cells were incubated for 15 min. After incubation, the aggregates were washed and mounted on slides for observation using a Zeiss Axioskop and AxioCam HRc digital camera (Carl Zeiss, Thornwood, NY). The circumference of the aggregates was measured by AxioVision V 4.5 software (Carl Zeiss). The number of sperm bound to the periphery of each aggregate was counted and

normalized by the aggregate circumference. Experiments were performed in triplicate and data analyzed by the PROC MIXED procedure in SAS (SAS Institute Inc., Cary, NC). The treatment was considered a fixed factor and replicate were considered a random factor. Tukey–Kramer's test was used for multiple comparisons between treatments. Differences were considered significant at *P-value* < 0.05.

3. **Results and discussion**

Although Lewis X dimers and trimers bind sperm with higher affinity, only Lewis X monomers were detected in the oviduct (Kadirvel et al. 2012). We used sulfated Lewis^X (sLe^X), which binds sperm with high affinity, to identify Lewis X-binding proteins in noncapacitated boar sperm by a combination of approaches that included glycan affinity chromatography, glycan blot analysis and LC-ESI-MS/MS.

3.1. Purification of sulfated Lewis X-binding proteins

We demonstrated that sLe^X covalently attached to Sepharose beads bound a cohort of proteins which were eluted using an EDTA/NaCl buffer. Buffer was added to the column until no more protein was detected in the flow-through fractions, by protein assay and by SDS-PAGE (Figure 4.2A and 4.2B) and then EDTA/NaCl was used to elute bound protein. Based on glycan blotting assay, at least 11 sLe^X-binding proteins were detected in eluted fractions E1 and E2. The greatest signal was found in a doublet at 14 kDa (Figure 4.3A). Only 5 of the sLe^X-binding proteins were adequately abundant to be detected by Coomassie Blue staining. The molecular weight (MW) of the proteins detected by Coomassie staining matched with the proteins identified as sLe^X-binding proteins by blotting assay (Figure 4.3B) and were approximately 14,

27, 44, 56 and 100 kDa. The five protein bands detected were excised and prepared for LC-MS/MS analysis.

3.2. LC-ESI-MS/MS analysis

A total of 22 unique reviewed proteins were identified (Table 4.1). A database search for unreviewed proteins was also included (Table 4.4). Gene ontology analysis of the proteins identified showed that 53% were associated with binding (Figure 4.8).

Based on the number of candidate proteins identified for each gel band, it was assumed that sLe^X-binding proteins were not isolated until apparent homogeneity. The mild detergent NP-40 was used during the purification/elution process and it is not expected to disrupt protein-protein interactions during chromatography. NP-40 was selected because detergent composition or concentration may dissociate protein-glycan interaction (Lotan et al. 1977) and minimize nonspecific interactions (Wei et al. 2010). Thus, the cytoplasmic proteins identified are likely originated from protein complexes formed after cell lysis and not disrupted until SDS-PAGE. A second glycan affinity column corroborated the presence of more than 60% of the proteins identified as P14, P27, P44 and P56 (Figure 4.9). Eluted fractions collected in this replicate identified a sixth protein that migrated as 87 kDa (Figure 4.10). A complete list of the proteins identified in a second affinity column replicate is also available (Table 4.5).

3.3. Protein Identity

P14. All the reviewed proteins identified, with the exception of histone H4, are extracellular or present in the acrosomal region. With the exception of histone H4 and sperm-associated acrosin inhibitor, all the proteins are involved with sperm capacitation or fertilization (Table 4.1). The

proteins identified as P14 belong to the seminal plasma protein family (seminal plasma protein pB1) and the spermadhesin family (AWN, AQN1, AQN3, seminal plasma sperm motility inhibitor) and have a MW of approximately 14 kDa. Spermadhesin proteins have heparin binding function, indicating the presence of a carbohydrate binding domain (CBD). The heparin binding function of these proteins is known to interact with the oviduct epithelium (Liberda et al. 2006). However, there are no previous reports that these proteins interact with Le^X structures.

Spermadhesin proteins are produced in the epididymis and are highly abundant in accessory gland fluids. They bind to the sperm surface during passage through the epididymis (AWN) or at ejaculation, when sperm are exposed to accessory gland fluids (AQN1, PSPI and II) coating the membrane covering the acrosomal cap (Dostalova et al. 1994). The protein with highest Mascot score, seminal plasma protein pB1, forms a complex with AQN1 (Calvete et al. 1997) and it was likely purified associated to this complex. AQN1 recognizes glycoproteins with either galactose or oligomannose termini and has been proposed as a molecule responsible for oviduct reservoir formation (Ekhlasi-Hundrieser et al. 2005). It is possible that sperm AQN1 attaches to oviduct cells through recognition of mannose and also alternatively Le^X structures. The ability of a protein to recognize distinct glycans is not an unusual feature; a common receptor to Le^X structures in other tissues is the dendritic cell-specific ICAM-3-grabbing nonintegrin (DC-SIGN) that is known to have binding specificity for high mannose oligosaccharides and Le^X trisaccharides (Guo et al. 2004)

P27. A few proteins previously identified as a P14 (acrosin-binding protein, seminal plasma sperm motility inhibitor, AWN and seminal plasma protein pB1) also appeared as P27. However, epididymal sperm-binding protein 1 (ESBP1) with a MW of approximately 27 kDa had the

highest Mascot score and it is most likely P27. ESBP1 belongs to the seminal plasma protein family and binds to the sperm surface upon ejaculation (Ekhlasi-Hundrieser et al. 2007). This protein has phosphorylcholine-binding activity, the likely mechanism by which it binds cells. It is possible that the protein was purified associated to other abundant seminal plasma proteins. Lactadherin and acrosin proteins were identified and will be discussed in the next section. Within the list of unreviewed proteins, acrosin (accession I3LD62), AWN (accession F1RI21) and PSP-1 (accession F1RI 24) were also presented. SPACA1 (accession D5K8A9) was identified. Mice deficient in SPACA1 produce sperm with abnormal morphology (Fujihara et al. 2012).

P44. The reviewed proteins identified as P44 by LC-MS/MS have a MW of 41 to 50 kDa. The two proteins with the highest Mascot scores were lactadherin and acrosin. All the other proteins identified are cytoplasmic proteins and are likely contaminants. Acrosin is an acrosomal serine protease released during the acrosome reaction to facilitate sperm penetration through the zona pellucida (Dunbar et al. 1985) . This is a multifunctional protein that contributes to sperm binding to the oocyte zona pellucida through recognition of fucose and mannose structures (Topfer-Petersen and Henschen 1987). Since this protein is only present on the surface of acrosome-reacted sperm, it would not be expected to function in sperm-oviduct binding, which is restricted to acrosome-intact sperm. However, this result indicates an affinity of acrosin to sLe^X which is consistent with its affinity for sulfated glycans (Gaboriau et al. 2007).

Lactadherin is also known as SED1, MFGM, MFG-E8 and sperm surface protein SP47. The name SED1 refers to a *s*ecreted protein with Notch-like type II *E*GF repeats and C-terminal *d*iscoidin domains (Ensslin M. A. and Shur 2003). The EGF domain contains the arginine-

glycine-aspartate attachment site that is found in integrin ligands (Ruoslahti 1996). The discoidin domain binds a multiplicity of ligands including phospholipids, carbohydrates and proteins (Kiedzierska et al. 2007). In addition to being found among the reviewed proteins, an isoform of lactadherin was identified within the list of unreviewed proteins as the isoform B2CZF8 (Table 4.2). Tandem MS revealed 7 unique peptides (Table 4.3). The peptides GDVFTQYIcK, AGSAEYIKTFK and TFKVAYSSDGRK, in the unreviewed isoform, were seen with several PSMs each indicating that two distinct proteins were detected. In addition, both proteins were detected bound to a second glycan affinity column, confirming that both isoforms are found in P44 (data not shown). The peptide sequences identified by LC-MS/MS for both isoforms covered the discoidin domain region (Figure 4.4).

Other unreviewed proteins of interest were PKDREJ (accession=F1SM79), SPACA1 (accession=D5K8A9), ADAM3a (accession=A5A4F6) and fertilin beta FTNB (accession=Q866A8), all involved with sperm function or binding (Hao et al. 2002, Kim et al. 2006, Sutton et al. 2008).

P56 and P100. Within the list of reviewed proteins, with the exception of acrosin and lactadherin, the other candidates in this bands are cytoplasmic or proteins that do not contain carbohydrate binding domains. Within the list of unreviewed proteins, the only protein that is associated with the fertilization process is SPAM-1 (accession=Q8MI02), the sperm hyaluronidase that aids in sperm penetration through the cumulus cells surrounding the oocyte (Lathrop et al. 1990). It is possible that proteins detected as P56 and P100 were indirectly bound to the column through protein complex affinity; for instance, sperm acrosin inhibitors have been

detected in purification processes associated with AQN1, AQN3, AWN and PSP1 (Calvete et al. 1996b).

3.4. Detection of lactadherin in boar sperm

Due to the abundance of lactadherin in the Le^X affinity column bound fractions, we focused our attention on lactadherin. Lactadherin is secreted by the epididymal epithelium and attaches to the sperm plasma membrane during epididymal passage (Ensslin M. A. and Shur 2003). This protein has been previously characterized in boar sperm (Ensslin M. et al. 1998) and binds to the zona pellucida in pig (Ensslin M. et al. 1998) and mouse (Ensslin M. A. and Shur 2003). Male mice deficient in lactadherin have reduced litter size due to zona pellucida binding impairment (Ensslin M. A. and Shur 2003).

The presence of lactadherin on the sperm plasma membrane was investigated using a commercially available polyclonal antibody against the mouse protein. The antigen used to produce the mouse antibody (residues Ala23-Cys463) showed 69% identity with the corresponding region of porcine lactadherin. Recombinant mouse lactadherin, which has a MW of 67 kDa, was used as a positive control. As expected, mouse antibody recognized a 67 kDa protein in mouse epididymal sperm and 47 kDa protein in boar epididymal and ejaculated sperm (Figure 4.5); however, it was possible to detect a lower molecular weight protein in pig and mouse sperm in multiple western blot assays. The lower molecular weight protein could be an additional form of the lactadherin protein or a different protein with cross reactivity with the antibody. At least in mouse epididymal tissue, short and long forms of lactadherin have been reported (Raymond and Shur 2009) that would account for the smaller band.

3.5. Lactadherin is involved in sperm binding to oviduct cells

When recombinant mouse lactadherin (rLac), which has a MW of 67 kDa, was resolved in one-dimensional SDS-PAGE and subsequently blotted with biotinylated sLe^X, a band of approximately 60 kDa was detected confirming that sLe^X binds to lactadherin (Figure 4.6A). It has been suggested that lactadherin binds to negatively charged glycans containing a sialic acid or sulfate group (Shur 2008); therefore, it is possible that lactadherin recognizes the sulfate residue of the Le^X motif. Glycan blot results show that rLac also binds to biotinylated Le^X demonstrating that recognition of sLe^X is not restricted to the negative sulfate residue (Figure 4.6B). In addition, since proteins with discoidin domains bind galactose (Kiedzierska et al. 2007), rLac also demonstrated affinity to *N*-acetyllactosamine by glycan blotting (Figure 4.6B). This is the first direct evidence that lactadherin binds Le^X structures and glycans with galactose residues.

To determine the biological importance of lactadherin, we tested its involvement in sperm-oviduct binding. The latter was accomplished by incubation of oviduct cell aggregates with recombinant lactadherin to occupy binding sites in oviduct cells. Pre-incubation with lactadherin reduced sperm binding to oviduct cells by 56.6% (Figure 4.6C) supporting the hypothesis that lactadherin at least partially mediates sperm binding. It has been previously suggested that lactadherin facilitates sperm binding to integrins on the oviduct surface (Petrunkina et al. 2003), but this is the first time that involvement of this protein in oviduct cell binding was demonstrated directly. However, increasing concentrations of lactadherin were not sufficient to completely inhibit sperm binding to cell aggregates suggesting the involvement of secondary adhesive proteins. Since multiple sperm proteins appear to recognize Le^X, it is possible that these other putative receptors are also involved in sperm-oviduct adhesion.

4. Conclusions

Oviduct Le^X binds sperm and this binding is required for normal sperm-oviduct adhesion to form a sperm reservoir. To identify putative Le^X receptors, a glycan affinity column was used that separated a cohort of sulfated Le^X-binding proteins from sperm. The proteins in the bands labeled as P14, P27 and P44 are closely related and accomplish similar functions associated with sperm binding. The presence of multiple sLe^X-binding proteins provides support to our previous observation that a complex system mediates sperm binding to oviduct cells (Silva et al. 2013).

Lactadherin, a protein largely known to be involved in cell-cell adhesion, was identified as the major component of P44. The unreviewed protein B2CZF8, a lactadherin isoform, was also identified. We confirmed that recombinant mouse lactadherin bound to sLe^X and that this protein competed with the sperm for binding sites in oviduct cells. Because only partial binding inhibition was achieved when cells were pre incubated with lactadherin, other proteins are perhaps involved in this binding system. Further studies are necessary to investigate if lactadherin recognizes the negatively charged sialylated *N*-acetyllactosamine motif which is an important glycan involved in sperm-oviduct binding.

In summary, we have identified multiple sperm proteins with sLe^X affinity and we have found that lactadherin has affinity for sLe^X motif and an important role in sperm-oviduct cell binding.

FIGURES AND TABLES

FIGURE 4.1.

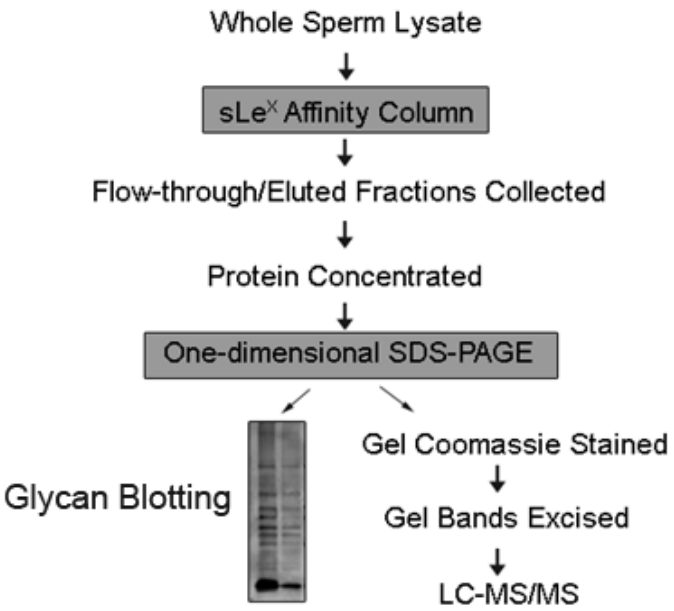

Figure 4.1. A schematic of the strategy used to identify sLe^X -binding proteins in whole sperm lysate. Two purification steps were used: 1) sLe^X affinity chromatography and 2) one-dimensional SDS-PAGE (gray boxes). Protein fractions recovered from the affinity column were concentrated and further separated by one-dimensional SDS-PAGE. The presence of sulfated Lewis X-binding proteins was confirmed by glycan blotting assays. A duplicate gel was stained with Coomassie Blue; the migration of the proteins identified were matched to glycan blot results. Protein bands corresponding to bands that bound glycans were excised from the gel and submitted to LC-MS/MS.

FIGURE 4.2.

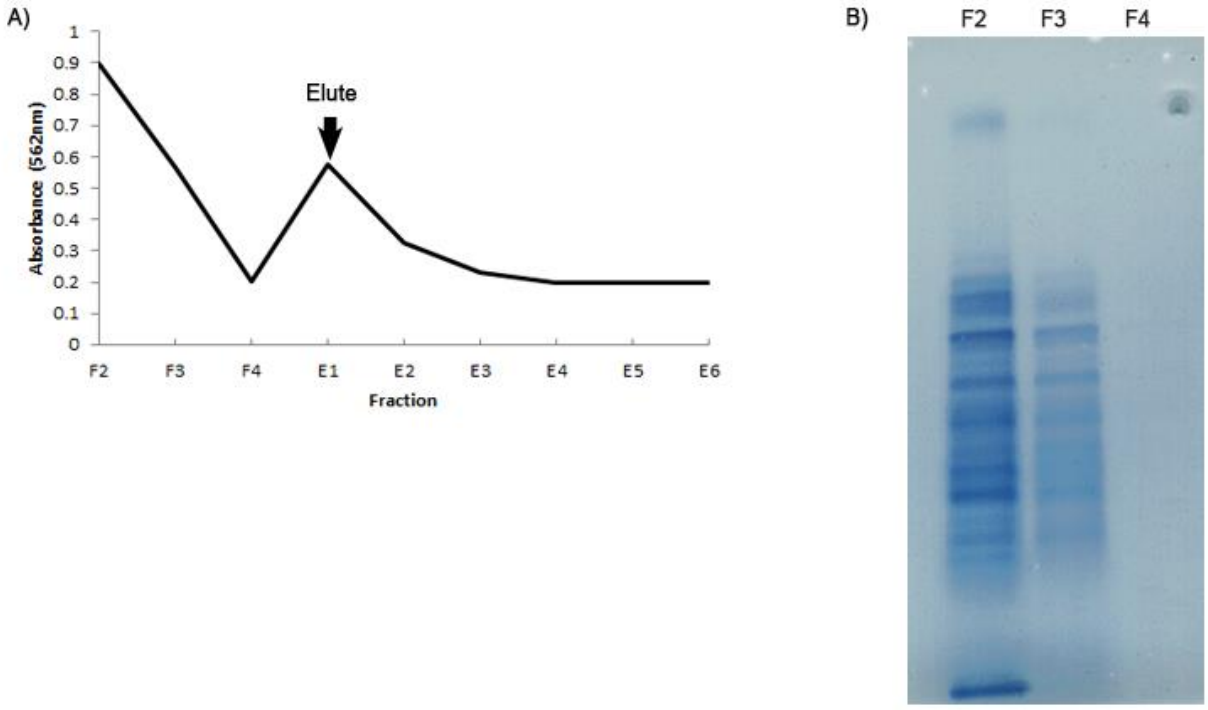

Figure 4.2. Protein profile of fractions collected from a sLe^X column. Whole sperm lysates were incubated with sLe^X covalently attached to Sepharose beads. The suspension was transferred to a minicolumn and a sequence of flow-through fractions (F1, F2, F3, F4) was collected. Protein was eluted with an EDTA/NaCl buffer. A) Protein concentration determined by BCA assay (F1 not shown). The black arrow indicates when first eluted fraction (E1) was collected. B) SDS-PAGE gel stained with Coomassie Blue to show protein profile of the last three flow-through fractions. A gradual reduction of protein in the flow through fractions was observed, demonstrating that the minicolumn was washed completely.

FIGURE 4.3.

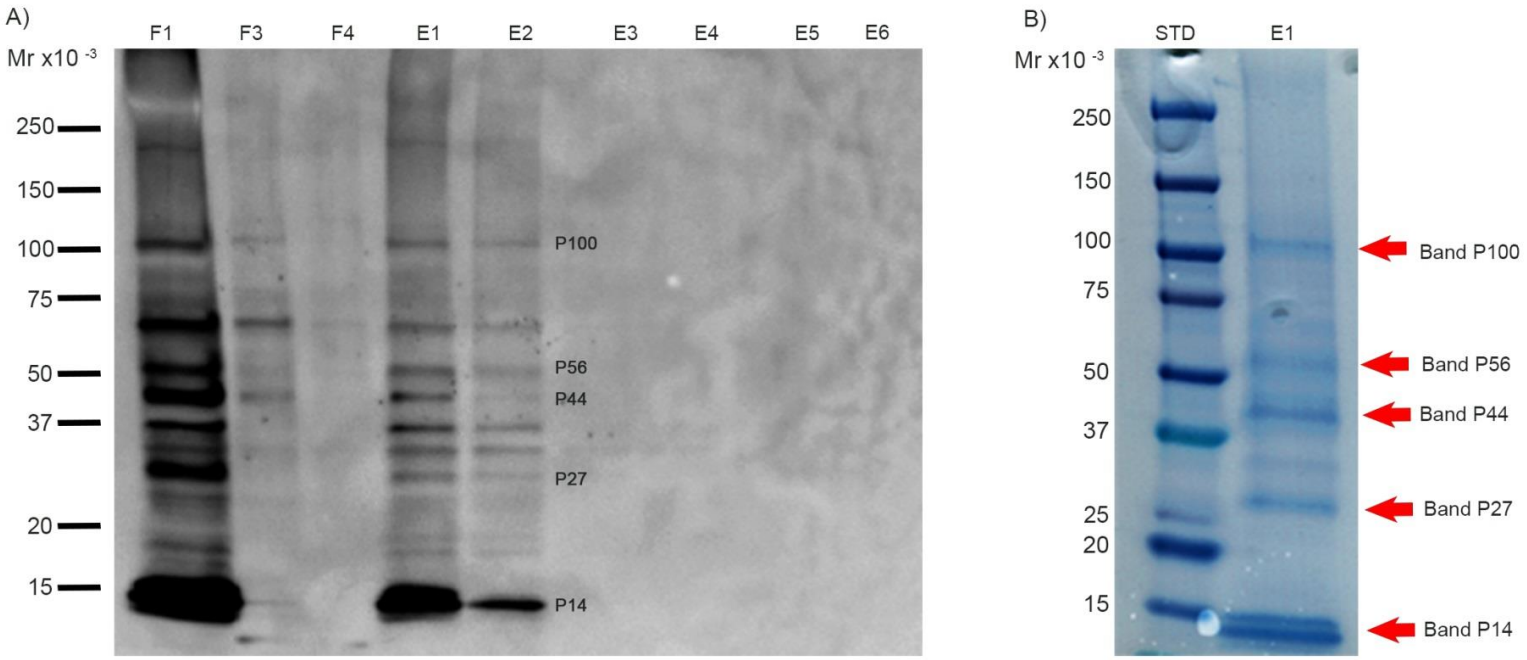

Figure 4.3. Glycan blotting assay and protein profile of fractions collected after sLe^X affinity chromatography. A) Equal volumes of protein eluates were separated by one-dimensional SDS-PAGE, transferred to nitrocellulose and probed with biotinylated sLe^X and avidin-HRP. sLe^X binding proteins were detected in the flow-through (F1, F3 and F4) and eluted (E1 –E6) fractions. B) The first eluted fraction was resolved by SDS PAGE and stained with Coomassie Blue. Clear distinct protein bands were excised from the gel and submitted to LC-MS/MS. Migration of molecular size markers is shown on the left of the figures.

FIGURE 4.4.

MFGE8 (reviewed protein)

```
  1  FSGDFCDSSLCLNGGTCLLDQDPQKPFHCLCPEGFTGLICNETEKGPCFPNPCHNDAECE
 61  VIDDAHRGDVFTEYICKCPHGYTGIHCEIICNAPLGMETGAIADFQISASSMHLGFMGLQ
121  RWAPELARLHRAGIVNAWTASNYDRNPWIQVNLLRRMRVTGVVTQGASRAGSAEYMKTFK
181  VAYSTDGRKFQFIQGAEESGDKIFMGNLDNSGLKVNLFEVPLEVQYVRLVPIICHRGCTL
241  RFELLGCELSGCAEPLGLKDNTIPNKQITASSFYRTWGLSAFSWYPFYARLDNQGKFNAW
301  TAQSNSASEWLQIDLGSQRRVTGIITQGARDFGHIQYVAAYKVAYSDDGVSWTEYRDQGA
361  LEGKIFPGNLDNNSHKKNMFETPFLTRFVRILPVAWHNRITLRVELLGC
```

B2CZF8 (unreviewed protein)

```
  1  MPGPRLLTAICGALLCASGLFAFSGDFCDSSQCLNGGTCLLDQDPQNPFHCLCPEGFTGL
 61  ICNETEKGPCFPNPCHNDAECEVIDDAHRGDVFTQYICKCPHGYTGIHCEIICNAPLGME
121  TGAIADFQISASSMHLGFMGLQRWAPELARLHRAGIVNAWTASNYDRNPWIQVNLLRRMR
181  VTGVVTQGASRAGSAEYIKTFKVAYSSDGRKFQFIQGAEESGDKIFMGNLDNSGLKVNLF
241  EVPLEVQYVRLVPIICHRGCTLRFELLGCELSGCAEPLGLKDNTIPNKQITASSFYRTWG
301  LSAFSWYPFYARLDNQGKFNAWTAQSNSASEWLQIDLGSQRRVTGIITQGARDFGHIQYV
361  AAYKVAYSDDGVSWTEYRDQGALEGKIFPGNLDNNSHKKNMFETPFLTRFVRILPVAWHN
401  RITLRVELLGC
```

Figure 4.4. Amino acid sequence of the two lactadherin isoforms identified by LC-MS/MS. Arrows indicate the putative starting point of the discoidin domain. Peptide sequences deduced by LC-MS/MS sequencing are shown in bold type.

FIGURE 4.5.

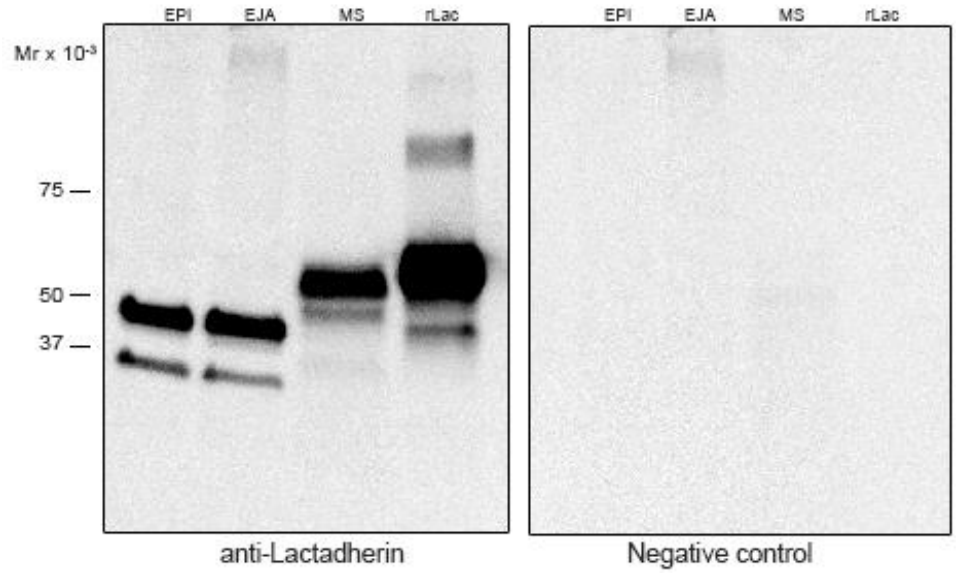

Figure 4.5. Immunoblot of boar sperm plasma membrane samples with lactadherin antibody. Proteins from boar epididymal (EPI), boar ejaculated (EJA) and mouse epididymal (MS) sperm were separated by SDS-PAGE and submitted to western blot analysis using anti-mouse lactadherin. Data show cross reactivity of mouse antibody with boar sperm. As a positive control, recombinant mouse lactadherin (rLac) was used. Negative control, where primary antibody was omitted, is shown on the right.

FIGURE 4.6.

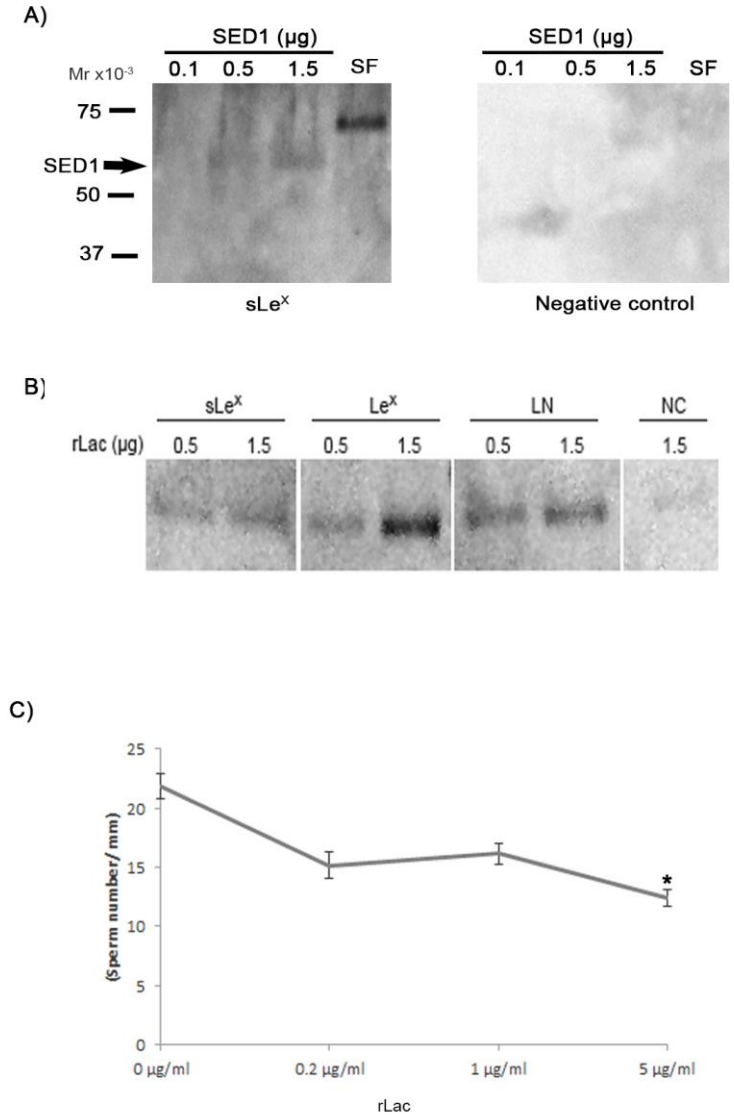

Figure 4.6. Biological activity of lactadherin, assessed by sLe^X blotting assay and competitive sperm binding assay. A) Increasing concentrations (0.1, 0.5 and 1.5 µg) of recombinant mouse lactadherin (rLac) were resolved on SDS-PAGE and subsequently incubated with biotinylated sLe^X. Seminal fluid (SF) was used as a positive controls. The black arrow indicates lactadherin signals in 0.5 and 1.5 µg lanes. The expected molecular weight of rLac is 67 kDa based on the manufacturer's information. Negative control, where the presence of biotinylated glycan was omitted, is shown on the right. B) Increasing concentrations (0.5 and 1.5 µg) of rLac were resolved on SDS-PAGE and subsequently incubated with biotinylated sLe^X, Le^X and LN. Negative control (NC) is shown on the right. C) Inhibition of sperm binding to oviduct cells incubated with rLac. Asterisks indicates treatment that had reduced number of sperm bound to oviduct aggregates compared to control treatment ($P < 0.01$).

FIGURE 4.7.

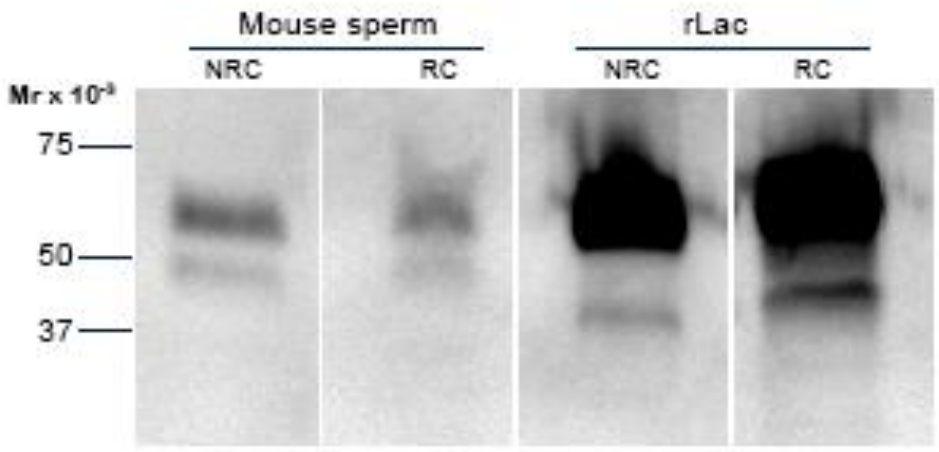

Figure 4.7. Detection of lactadherin in epididymal mouse sperm (15 µg) and recombinant mouse lactadherin (rLac; 200 ng) by western blot. Protein samples were resolved on SDS-PAGE under reducing conditions (RC) and non reducing conditions (NRC) and subsequently incubated with mouse lactadherin antibody.

FIGURE 4.8.

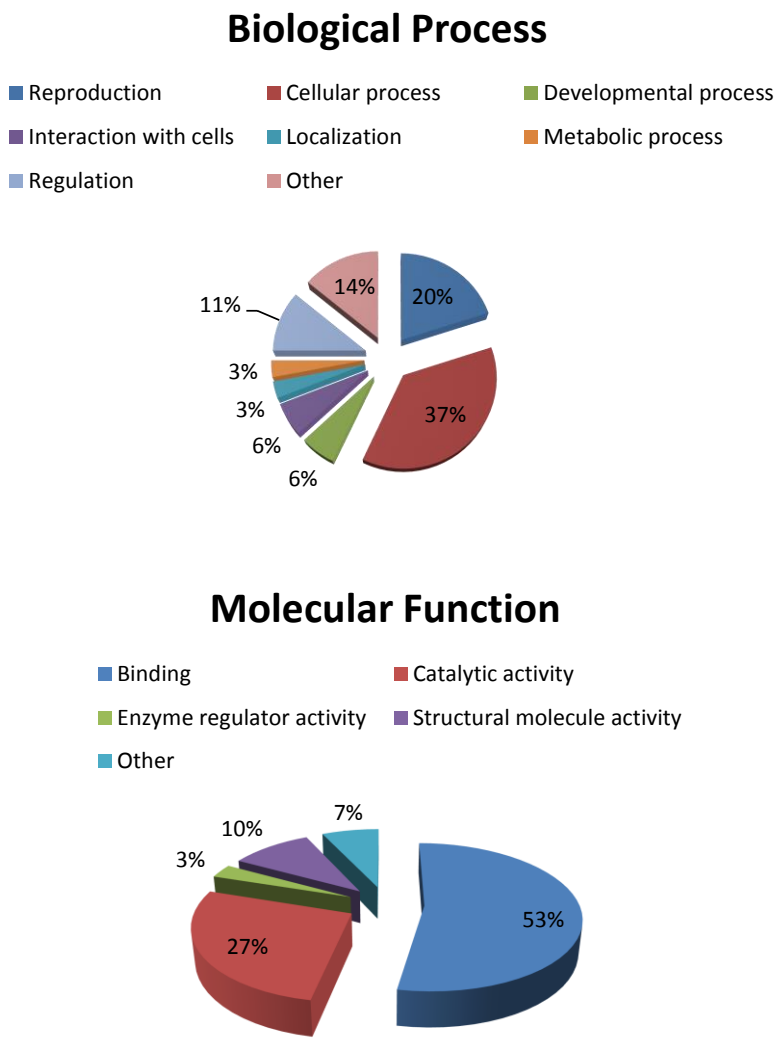

Figure 4.8. Gene ontology annotations of proteins retained by a sLe^X affinity column and identified by mass spectrometry analysis.

FIGURE 4.9.

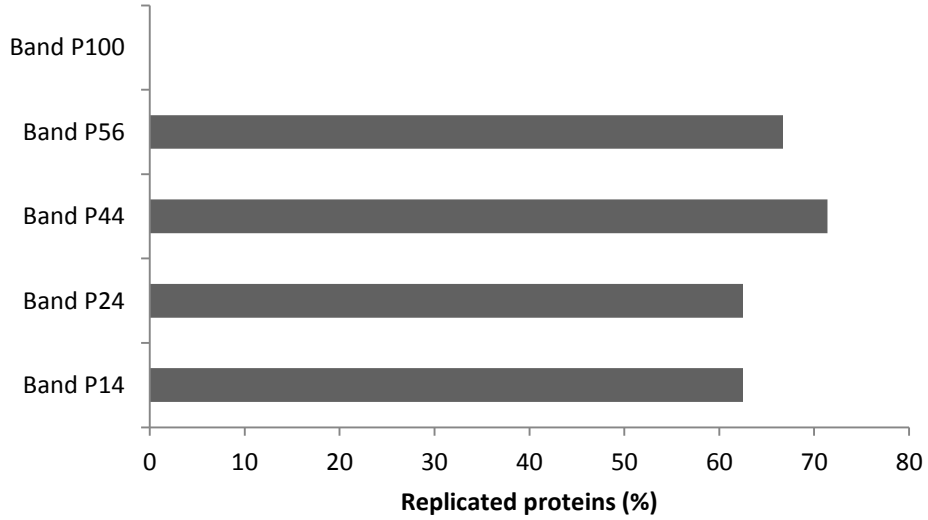

Figure 4.9. Percentage of proteins identified in the first affinity column that were identified in a second experimental replicate.

FIGURE 4.10.

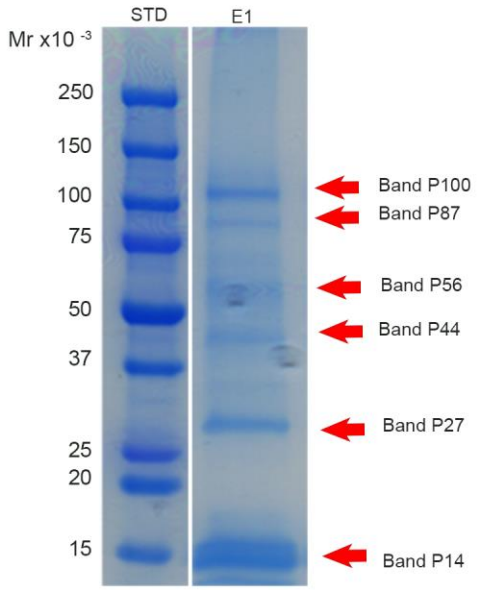

Figure 4.10. Protein profile of eluted fraction collected after a second replicate of sLe^X affinity column. After protein separation by SDS-PAGE, gel was stained by Coomassie Blue. The migration of molecular markers showed on the left. Red arrows indicate gel bands that were excised and subjected to mass spectrometry analysis. Only proteins with clear distinct bands were excised. An extra band (P87) was excised in this replicate.

Table 4.1. Identification of sperm proteins retained in a sulfated Lewis X affinity column by LC-MS/MS analysis (list of reviewed proteins)[1].

Accession[2]	Protein name[3]	MW (kDa)	Mascot score[4]	Function[5]	Biological Process[5]	Component[5]	CBD[6]	Rep[7]
Band P14								
P80964	Seminal plasma protein pB1	15.36	395.82	-	Sperm capacitation	Extracellular region	No	✓
P26776	**Carbohydrate-binding protein AWN**	**16.72**	**366.44**	**Heparin binding**	**Single fertilization**	**Extracellular region**	**Yes**	✓
P26322	**Carbohydrate-binding protein AQN-1**	**11.87**	**325.56**	**Heparin binding**	**Single fertilization**	**Extracellular region**	**Yes**	✓
P62802	Histone H4	11.36	287.42	DNA binding	Nucleosome assembly	Nucleus	No	✓
P24020	**Carbohydrate-binding protein AQN-3**	**12.88**	**269.96**	**Heparin binding**	**Single fertilization**	**Extracellular region**	**Yes**	✓
Q28920	**Seminal plasma sperm motility inhibitor**	**15.19**	**205.46**	**Heparin binding**	**Single fertilization**	**Extracellular region**	**Yes**	
P26461	Sperm-associated acrosin inhibitor	11.13	203.59	Serine type endopeptidase inhibitor	Negative regulation peptidase activity	Extracellular region	No	
Q29016	Acrosin-binding protein (Fragment)	60.50	129.79	Protein binding	Sperm capacitation	Acrosomal region	No	
Band P27								
Q7YR83	Epididymal sperm-binding protein 1	26.18	598.12	-	Single fertilization	Extracellular region	No	✓
Q29016	Acrosin-binding protein (Fragment)	60.50	297.31	Protein binding	Sperm capacitation	Acrosomal region	No	✓
P79385	**Lactadherin**	**45.70**	**124.86**	**-**	**Single fertilization /Cell adhesion**	**Acrosomal membrane**	**Yes**	✓
Q6QRN9	ADP/ATP translocase 3	32.89	82.5	Transport	Transporter activity	Mitochondrial membrane	No	

Table 4.1. *(continued)*

Accession[1]	Protein name[3]	MW (kDa)	Mascot score[4]	Function[5]	Biological Process[5]	Component[5]	CRD[6]	Rep[7]
Band P27								
Q28920	**Seminal plasma sperm motility inhibitor**	**15.19**	**78.22**	**Heparin binding**	**Single fertilization**	**Extracellular region**	**Yes**	✓
P08001	**Acrosin**	**45.33**	**74.31**	**Fucose/Mannose binding**	**Single fertilization**	**Acrosomal vesicle**	**Yes**	✓
P26776	**Carbohydrate-binding protein AWN**	**16.72**	**67.02**	**Heparin binding**	**Single fertilization**	**Extracellular region**	**Yes**	✓
P80964	Seminal plasma protein pB1	15.36	57.07	-	Sperm capacitation	Extracellular region	No	
Band P44								
P79385	**Lactadherin**	**45.70**	**1364.94**	**-**	**Single fertilization /Cell adhesion**	**Extracellular region**	**No****	✓
P08001	**Acrosin**	**45.33**	**521.9**	**Fucose/Mannose binding**	**Single fertilization**	**Acrosomal vesicle**	**Yes**	✓
Q6RI85	Phosphoglycerate kinase 2	44.87	211.45	Nucleotide binding	Glycolysis/Phosphorylation	Cytoplasm	No	
O97580	Succinyl-CoA ligase subunit beta, mitochondrial (Fragment)	46.23	174.65	ATP binding	TCA cycle	Mitochondrion	No	
Q6QAQ1	Actin, cytoplasmic 1	41.71	133.09	ATP binding	-	Cytoplasm	No	
P62197	26S protease regulatory subunit 8	45.60	94.87	Nucleotide binding	Protein catabolic process	Nucleus	No	
Q2XVP4	Tubulin alpha-1B chain	50.12	86.36	Structural constituent of cytoskeleton	Microtubule cytoskeleton organization	Cytoplasm	No	✓

Table 4.1. *(continued)*

Accession[2]	Protein name[3]	MW (kDa)	Mascot score[4]	Function[5]	Biological Process[5]	Component[5]	CRD[6]	Rep[7]
Band P56								
P02554	Tubulin beta chain	49.83	502.7	Protein binding	Protein polymerization	Cytoplasm	No	✓
P08001	**Acrosin**	**45.33**	**319.29**	**Fucose/Mannose binding**	**Single fertilization**	**Acrosomal vesicle**	**Yes**	✓
P02550	Tubulin alpha-1A chain	50.04	304.32	Constituent of cytoskeleton	Microtubule-based process	Cytoplasm	No	
Q29236	T-complex protein 1 subunit zeta (Fragment)	10.93	192.91	Unfolded protein binding	Protein folding	Cytoplasm	No	✓
Q29596	ATP synthase subunit alpha liver isoform, mitochondrial (Fragment)	15.56	150.54	ATP binding	ATP biosynthetic process	Mitochondrion	No	✓
F1RKQ4	Bifunctional ATP-dependent dihydroxyacetone kinase/FAD-AMP lyase (cyclizing)	59.20	80.39	ATP binding	Metabolic process	-	No	✓
Q8WNW3	Junction plakoglobin	81.80	73.32	Protein phosphatase binding	Cell adhesion	Nucleus/Cytoplasm/ Plasma membrane	No	
P79385	**Lactadherin**	**45.70**	**70.82**	**-**	**Single fertilization /Cell adhesion**	**Extracellular region**	**No**[**]	✓
Q6QAQ1	Actin, cytoplasmic 1	41.71	58.29	ATP binding	-	Cytoplasm	No	
Band P100								
Q6QAQ1	Actin, cytoplasmic 1	41.71	74.83	ATP binding	-	Cytoplasm	No	

[1]Proteins retained in a sLe^X affinity column were submitted to one-dimensional SDS-PAGE. After protein separation, gel was stained with Coomassie Blue and protein bands were excised. Gel bands were trypsin digested and analyzed via LC-MS/MS on a Bruker amaZon ETD ion trap using CID for fragmentation.
[2and3] Data from UniProt databases. Data included proteins with Mascot score ≥ 50 and with at least 2 peptides detected.
[4and 5]Database was searched using the Mascot search engine on the Matrix Science public domain server (www.matrixscience.com). Protein function, biological processes and component information were obtained from UniProtKB-GOA QuickGO (Binns et al. 2009).
[6]Presence of carbohydrate recognition domain (CRD).
[7]Proteins that were identified in a second technical replicate of the glycan affinity column.
[**] The discoidin domain of lactadherin is known to have galactose affinity (Kiedzierska et al. 2007).

Table 4.2. Characteristics of the two lactadherin isoforms identified by LC-MS/MS.

Accession[1]	Description[1]	MW [kDa] [1]	Score[2]	Coverage[2]	Unique Peptides[2]	Peptides[2]	PSMs[3]	Amino acids
B2CZF8	Lactadherin [B2CZF8_PIG]	47.8	1876.545	58.9	7	36	860	431
P79385	Lactadherin [MFGM_PIG]	45.7	1796.282	58.9	1	30	824	409

[1]Data from UniProt databases.

[2]Database was searched using Mascot (Matrix Science).

[3]PSM= peptide spectral matches.

Table 4.3. Peptide sequences of lactadherin B2CZF8 and P79385 by LC-MS/MS analysis

Peptide Sequence	MH+ [Da]	Mass Error [ppm]	XCorr	# PSMs
B2CZF8				
VAYSDDGVSWTEYRDQGALEGK	2446.115	-0.4	7.27	38
KFQFIQGAEESGDK	1583.767	-1.9	5.08	15
DFGHIQYVAAYK	1411.699	-1.1	4.77	87
DNTIPNKQITASSFYR	1854.933	-0.8	4.67	14
AGIVNAWTASNYDR	1537.736	-2.3	4.66	18
FELLGcELSGcAEPLGLK	1992.978	0.3	4.59	6
RVTGIITQGAR	1171.687	-2.8	4.56	23
MRVTGVVTQGASR	1377.727	0.5	4.50	22
VAYSDDGVSWTEYR	1647.728	-0.4	4.40	19
IFPGNLDNNSHKK	1483.769	2.6	4.29	40
TFKVAYSSDGRK*	**1358.707**	**0.8**	**4.29**	**3**
GPcFPNPcHNDAEcEVIDDAHR	2610.052	-1.5	4.21	4
VNLFEVPLEVQYVR	1704.930	-1.0	4.15	29
FELLGcELSGcAEPLGLKDNTIPNK	2775.370	0.2	4.09	1
MRVTGVVTQGASR	1361.732	-0.2	4.00	1
FQFIQGAEESGDK	1455.673	-1.4	3.77	16
VTGVVTQGASR	1074.587	-3.4	3.67	14
FQFIQGAEESGDKIFmGNLDNSGLK	2761.320	2.2	3.65	2
AGSAEYIKTFK*	**1214.641**	**-0.5**	**3.51**	**3**
IFPGNLDNNSHK	1355.672	1.3	3.37	22
IFmGNLDNSGLK	1324.657	0.2	3.34	30
KNmFETPFLTR	1399.707	2.3	3.29	13
IFMGNLDNSGLK	1308.662	0.1	3.25	4
NmFETPFLTR	1271.613	3.5	3.22	171
TFKVAYSSDGR*	**1230.610**	**-1.3**	**3.22**	**4**
VTGIITQGAR	1015.591	1.6	3.22	98
KFQFIQGAEESGDKIFmGNLDNSGLK	2889.412	1.0	3.21	3
ILPVAWHNR	1105.629	2.0	3.19	23

Table 4.3. *(continued)*

Peptide Sequence	MH+ [Da]	Mass Error [ppm]	XCorr	# PSMs
B2CZF8				
NPWIQVNLLR	1252.717	0.7	3.04	3
NMFETPFLTR	1255.617	2.5	3.03	5
QITASSFYR	1072.546	3.4	2.98	23
GDVFTQYIcK*	**1230.585**	**2.3**	**2.76**	**13**
LVPIIcHR	1007.580	-1.6	2.64	21
AGSAEYIK*	**838.428**	**-2.5**	**2.63**	**2**
DQGALEGK	817.404	-1.4	2.36	12
VAYSSDGR*	**854.400**	**0.0**	**2.33**	**6**
WAPELAR	842.452	-0.4	2.15	42
VAYSSDGRK*	**982.494**	**-1.3**	**2.08**	**6**
DNTIPNK	801.410	-0.3	1.71	4
P79385				
VAYSDDGVSWTEYRDQGALEGK	2446.115	-0.4	7.27	38
KFQFIQGAEESGDK	1583.767	-1.9	5.08	15
DFGHIQYVAAYK	1411.699	-1.1	4.77	87
DNTIPNKQITASSFYR	1854.933	-0.8	4.67	14
AGIVNAWTASNYDR	1537.736	-2.3	4.66	18
FELLGcELSGcAEPLGLK	1992.978	0.3	4.59	6
RVTGIITQGAR	1171.687	-2.8	4.56	23
mRVTGVVTQGASR	1377.727	0.5	4.50	22
VAYSDDGVSWTEYR	1647.728	-0.4	4.40	19
IFPGNLDNNSHKK	1483.769	2.6	4.29	40
GPcFPNPcHNDAEcEVIDDAHR	2610.052	-1.5	4.21	4
VNLFEVPLEVQYVR	1704.930	-1.0	4.15	29
FELLGcELSGcAEPLGLKDNTIPNK	2775.370	0.2	4.09	1
MRVTGVVTQGASR	1361.732	-0.2	4.00	1

Table 4.3. *(continued)*

Peptide Sequence	MH+ [Da]	Mass Error [ppm]	XCorr	# PSMs
P79385				
FQFIQGAEESGDK	1455.673	-1.4	3.77	16
VTGVVTQGASR	1074.587	-3.4	3.67	14
FQFIQGAEESGDKIFmGNLDNSGLK	2761.320	2.2	3.65	2
IFPGNLDNNSHK	1355.672	1.3	3.37	22
IFmGNLDNSGLK	1324.657	0.2	3.34	30
KNmFETPFLTR	1399.707	2.3	3.29	13
IFMGNLDNSGLK	1308.662	0.1	3.25	4
NmFETPFLTR	1271.613	3.5	3.22	171
VTGIITQGAR	1015.591	1.6	3.22	98
KFQFIQGAEESGDKIFmGNLDNSGLK	2889.412	1.0	3.21	3
ILPVAWHNR	1105.629	2.0	3.19	23
NPWIQVNLLR	1252.717	0.7	3.04	3
NMFETPFLTR	1255.617	2.5	3.03	5
QITASSFYR	1072.546	3.4	2.98	23
GDVFTEYIcK*	**1231.559**	**-6.3**	**2.77**	**1**
LVPIIcHR	1007.580	-1.6	2.64	21
DQGALEGK	817.404	-1.4	2.36	12
WAPELAR	842.452	-0.4	2.15	42
DNTIPNK	801.410	-0.3	1.71	4

*Unique peptides identified.

Table 4.4. List of sperm proteins retained by a sLe^X affinity column and identified by mass spectrometry analysis (list of unreviewed proteins)[1].

Accession[2]	Protein Description[3]	MW (kDa)	Mascot score[4]	Biological Process[5]	Component[5]
Band P14					
Q4R0H8	**Spermadhesin AWN OS=Sus scrofa GN=AWN PE=2 SV=1**	**16.89**	**412.92**	**Single fertilization**	**-**
I7HJH6	**Spermadhesin AQN-3 OS=Sus scrofa GN=AQN-3 PE=2 SV=1**	**15.02**	**376.34**	**Single Fertilization**	**-**
I3LVF9	Uncharacterized protein OS=Sus scrofa GN=VAMP3 PE=4 SV=1	17.30	193.13	Vesicle-mediated transport	Membrane
F1SND1	Uncharacterized protein (Fragment) OS=Sus scrofa GN=TXNDC8 PE=4 SV=2	8.54	95.43	Cell redox homeostasis	-
F1SD28	Uncharacterized protein (Fragment) OS=Sus scrofa PE=4 SV=2	7.65	82.09	-	
F2Z5L5	Histone H2A OS=Sus scrofa GN=HIST2H2AC PE=3 SV=1	13.98	79.31	Nucleosome assembly	Nucleus
F1RM58	Uncharacterized protein OS=Sus scrofa GN=Ssc.92096 PE=4 SV=2	12.41	72.35	Response to biotic stimulus	Integral to membrane
I3LG15	Solute carrier family 2, facilitated glucose transporter member 3 (Fragment) OS=Sus scrofa GN=SLC2A3 PE=3 SV=1	53.79	62.52	Transport	Membrane
Q29574	Histone H2B (Fragment) OS=Sus scrofa PE=2 SV=1	7.62	58.13	Nucleosome assembly	Nucleus
F1RQP0	Uncharacterized protein OS=Sus scrofa GN=PRDX5 PE=4 SV=2	17.25	55.59	Response to oxidative stress	Cytoplasm
Band P27					
F1SL45	Acrosin-binding protein OS=Sus scrofa GN=ACRBP PE=4 SV=1	60.79	297.31	-	-
F1SGG9	Uncharacterized protein (Fragment) OS=Sus scrofa GN=Ssc.57524 PE=3 SV=2	60.03	214.04	-	Intermediate filament
F1SI89	Uncharacterized protein OS=Sus scrofa GN=TMEM202 PE=4 SV=2	31.23	140.09	-	-
D5K8A9	Sperm acrosome associated 1 OS=Sus scrofa GN=SPACA1 PE=2 SV=1	32.22	127.03	-	-

Table 4.4. *(continued)*

Accession[2]	Protein Description[3]	MW (kDa)	Mascot score[4]	Biological Process[5]	Component[5]
Band P27					
F1SB33	Uncharacterized protein OS=Sus scrofa GN=Ssc.1085 PE=4 SV=1	28.41	78.55	Transport	Membrane
F1RI25	Seminal plasma sperm motility inhibitor OS=Sus scrofa GN=SPMI PE=4 SV=1	14.65	78.22	-	-
I3LD62	**Acrosin (Fragment) OS=Sus scrofa GN=ACR PE=3 SV=1**	**27.86**	**74.31**	**Binding of sperm to zona pellucida**	**Acrosomal vesicle**
F1RI21	**Uncharacterized protein (Fragment) OS=Sus scrofa GN=AWN PE=4 SV=1**	**9.46**	**67.02**	**Single fertilization**	**-**
F1RP17	T-complex protein 1 subunit gamma OS=Sus scrofa GN=CCT3 PE=3 SV=2	61.07	63.36	Protein folding	Cytoplasm
F1RW75	Uncharacterized protein OS=Sus scrofa GN=DSP PE=4 SV=2	308.18	58.13	Adherens junction organization	Cell-cell junction
F1RI24	**Uncharacterized protein OS=Sus scrofa GN=PSP-I PE=4 SV=2**	**14.46**	**49.69**	**Single fertilization**	**-**
I3LQG1	Uncharacterized protein OS=Sus scrofa GN=GLIPR1L1 PE=4 SV=1	19.46	44.34	-	Extracellular region
I3LVM4	Uncharacterized protein OS=Sus scrofa GN=Ssc.47098 PE=4 SV=1	26.89	44.3	-	-
Band P44					
B2CZF8	**Lactadherin OS=Sus scrofa GN=MFGE8 PE=2 SV=1**	**47.82**	**1412.57**	**Cell adhesion**	**Extracellular space**
F1SN58	Uncharacterized protein OS=Sus scrofa GN=SQRDL PE=4 SV=2	50.11	446.95	Oxidation-reduction process	-
F2Z5I5	Uncharacterized protein OS=Sus scrofa GN=CSNK2A1 PE=4 SV=1	45.11	372.57	Protein phosphorylation	Nucleus
F1S8P1	Uncharacterized protein OS=Sus scrofa GN=Ssc.35552 PE=4 SV=2	47.46	370.6	Oxidation-reduction process	-

Table 4.4. *(continued)*

Accession[2]	Protein Description[3]	MW (kDa)	Mascot score[4]	Biological Process[5]	Component[5]
Band P44 continue					
F1SM79	**Uncharacterized protein OS=Sus scrofa GN=PKDREJ PE=4 SV=2**	**243.50**	**260.93**	**Acrosome reaction**	**Plasma membrane**
F1RK10	Succinyl-CoA ligase [ADP-forming] subunit beta OS=Sus scrofa GN=SUCLA2 PE=3 SV=2	50.27	233.69	Metabolic process	Mitochondrion
D5K8A9	Sperm acrosome associated 1 OS=Sus scrofa GN=SPACA1 PE=2 SV=1	32.22	210.84	-	-
Q8WNW8	Nexin-1 OS=Sus scrofa GN=PN-1 PE=2 SV=2	44.06	184.11	Negative regulation of cell growth	Cytosol, extracellular region
F1RK98	Uncharacterized protein OS=Sus scrofa GN=TUBA3E PE=3 SV=2	49.93	152.74	GTP catabolic process	Cytoplasm
Q28937	Beta actin (Fragment) OS=Sus scrofa PE=2 SV=1	26.10	133.09	Sarcomere organization	Cytoplasm
Q29220	Tubulin alpha-3 chain (Fragment) OS=Sus scrofa PE=2 SV=1	13.56	124.27	GTP catabolic process	Microtubule
A5A4F6	ADAM3a OS=Sus scrofa PE=2 SV=1	82.89	109.53	Proteolysis	-
F1STD5	Uncharacterized protein (Fragment) OS=Sus scrofa GN=ACTR1B PE=3 SV=2	40.48	97.62	-	Cytoplasm
Q866A8	**Fertilin beta OS=Sus scrofa GN=FTNB PE=2 SV=1**	**81.75**	**89.52**	**Cell adhesion[7]**	**-**
Band P56					
I3LCA2	Uncharacterized protein OS=Sus scrofa GN=CCT8 PE=3 SV=1	59.6	1159.93	Protein folding	Cytoplasm
F1RHU4	Uncharacterized protein (Fragment) OS=Sus scrofa GN=IL4I1 PE=4 SV=2	65.88	844.75	Oxidation-reduction process	Lysosome
D0G0C9	Chaperonin containing TCP1, subunit 7 (Eta) OS=Sus scrofa GN=CCT7 PE=2 SV=1	59.43	820.97	Protein folding	Cytoplasm

Table 4.4. *(continued)*

Accession[2]	Protein Description[3]	MW (kDa)	Mascot score[4]	Biological Process[5]	Component[5]
Band P56 continue					
F2Z571	Uncharacterized protein OS=Sus scrofa GN=TUBB4B PE=3 SV=1	49.79	723.74	GTP catabolic process	Nucleus/Cytoplasm
F1RIU3	T-complex protein 1 subunit zeta OS=Sus scrofa GN=CCT6 PE=3 SV=1	57.93	650.95	Protein folding	Cytoplasm
F1RP17	T-complex protein 1 subunit gamma OS=Sus scrofa GN=CCT3 PE=3 SV=2	61.07	481.41	Protein folding	Cytoplasm
F1RK98	Uncharacterized protein OS=Sus scrofa GN=TUBA3E PE=3 SV=2	49.92	382.84	GTP catabolic process	Cytoplasm
F1SGG9	Uncharacterized protein (Fragment) OS=Sus scrofa GN=Ssc.57524 PE=3 SV=2	60.03	341.96	-	Intermediate filament
F1SB63	T-complex protein 1 subunit alpha OS=Sus scrofa GN=TCP1 PE=3 SV=1	60.31	316.04	Protein folding	Cytoplasm
F1RPS8	ATP synthase subunit alpha (Fragment) OS=Sus scrofa GN=ATP5A1 PE=3 SV=1	57.59	225.56	ATP catabolic process	Mitochondrion
D0G0C8	Chaperonin containing TCP1, subunit 2 (Beta) OS=Sus scrofa GN=CCT2 PE=2 SV=1	57.40	167.43	Protein folding	Nucleus/cytoplasm
F1RXA7	Uncharacterized protein OS=Sus scrofa GN=PSMD3 PE=4 SV=2	61.14	156.67	Regulation catalytic activity	Proteasome complex
Q0QEM6	ATP synthase subunit beta (Fragment) OS=Sus scrofa GN=ATP5B PE=2 SV=1	47.05	155.08	ATP metabolic process	Mitochondrion
I3LR32	Uncharacterized protein OS=Sus scrofa GN=CCT5 PE=3 SV=1	54.48	148.57	Protein folding	Cytoplasm
F2Z5J1	Uncharacterized protein OS=Sus scrofa GN=Ssc.1911 PE=3 SV=1	49.15	139.74	Protein catabolic process	Cytoplasm
I3LT97	Uncharacterized protein OS=Sus scrofa GN=CCT6B PE=4 SV=1	25.56	112.61	Protein folding	-
F1S354	Membrane cofactor protein (Fragment) OS=Sus scrofa GN=CD46 PE=4 SV=2	43.88	106.2	-	-
Q8MI02	**Sperm adhesion molecule 1 OS=Sus scrofa GN=SPAM-1 PE=2 SV=1**	**56.00**	**99.47**	**Fusion sperm to egg**	**-**

Table 4.4. *(continued)*

Accession[2]	Protein Description[3]	MW (kDa)	Mascot score[4]	Biological Process[5]	Component[5]
Band 56 continue					
F1RW75	**Uncharacterized protein OS=Sus scrofa GN=DSP PE=4 SV=2**	**308.18**	**98.66**	**Adherens junction organization**	**Cell-cell junction**
C1PIG4	cAMP-dependent protein kinase regulatory subunit type II alpha OS=Sus scrofa GN=PRKAR2A PE=2 SV=1	45.09	81.86	Kinase activity	Cytoplasm
F1RKQ4	Bifunctional ATP-dependent dihydroxyacetone kinase/FAD-AMP lyase (cyclizing) OS=Sus scrofa GN=DAK PE=1 SV=2	59.19	80.39	Metabolic process	-
Q8WNW3	**Junction plakoglobin OS=Sus scrofa GN=Jup PE=2 SV=1**	**81.79**	**73.32**	**Cell adhesion**	**Cell-cell junction**
Q8SPK6	Beta-actin (Fragment) OS=Sus scrofa GN=beta-actin PE=3 SV=1	18.77	58.29	Sarcomere organization	Cytosol
I3LGN8	Uncharacterized protein OS=Sus scrofa GN=PKP1 PE=4 SV=1	51.54	53.18	Filament bundle assembly	Nucleus
F1RFZ6	Uncharacterized protein OS=Sus scrofa GN=C16ORF42 PE=3 SV=2	34.34	51.91	rRNA processing	-
I3LR45	Uncharacterized protein OS=Sus scrofa GN=MRPL9 PE=4 SV=1	30.30	49.76	Translation	Intracellular
F1RMZ8	V-type ATP synthase beta chain OS=Sus scrofa GN=ATP6V1B2 PE=3 SV=1	56.57	47.72	Transport	Proton- transporting complex
I3LLJ4	Uncharacterized protein (Fragment) OS=Sus scrofa PE=4 SV=1	33.14	42.21	Signal transduction	Intracellular
Band 100					
F1SGG9	Uncharacterized protein (Fragment) OS=Sus scrofa GN=Ssc.57524 PE=3 SV=2	60.03	169.19	-	Intermediate filament
Q8SPK6	Beta-actin (Fragment) OS=Sus scrofa GN=beta-actin PE=3 SV=1	18.77	74.83	Sarcomere organization	Cytosol

[1]Proteins retained by a sLe^X affinity column were submitted to a SDS-PAGE. After protein separation, gel was stained with Coomassie Blue and protein bands were excised and subjected to mass spectrometry analysis.

[2, 3 and 4]Data from UniProt databases. Data included proteins with Mascot score ≥ 50 and with at least 2 peptides. Database was searched using the Mascot search engine on the Matrix Science public domain server (www.matrixscience.com).

[5]Peptides identified.

[6]Protein function, biological processes and component information were obtained from UniProtKB-GOA QuickGO (Binns et al. 2009). Rows in bold refer to proteins related to the fertilization process and/or cell adhesion.

[7](Day et al. 2003).

Table 4.5. Replicate of mass spectrometry analysis of proteins separated by sLe^X affinity chromatography (list of reviewed proteins)[1].

Accession[2]	Protein name[2]	MW (kDa)	Mascot score[2]	Peptides[3]
Band P14				
P80964	Seminal plasma protein pB1	15.4	353.4	7
P26322	Carbohydrate-binding protein AQN-1	11.9	287.8	5
P26776	Carbohydrate-binding protein AWN	16.7	269	5
P24020	Carbohydrate-binding protein AQN-3	12.9	258.9	7
P35495	Major seminal plasma glycoprotein PSP-I	14.5	229.6	4
P62802	Histone H4	11.4	195	4
P00999	Seminal plasma acrosin inhibitor A1	7.6	121.8	3
Band P27				
Q7YR83	Epididymal sperm-binding protein 1	26.2	784.3	17
Q29016	Acrosin-binding protein (Fragment)	60.5	408.8	7
P35495	Major seminal plasma glycoprotein PSP-I	14.5	163.7	3
P26776	Carbohydrate-binding protein AWN	16.7	130.8	3
P26322	Carbohydrate-binding protein AQN-1	11.9	129.6	2
O77591	Inositol monophosphatase 1	30.1	126	2
P08001	Acrosin	45.3	113.4	2
Q28920	Seminal plasma sperm motility inhibitor	15.2	112.9	2

Table 4.5. *(continued)*

Accession[2]	Protein name[2]	MW (kDa)	Mascot score[2]	Peptides[3]
Band P44				
P79385	Lactadherin	45.7	1187.4	23
Q6RI85	Phosphoglycerate kinase 2	44.9	1074.9	25
P08001	Acrosin	45.3	401.3	8
Q6QAQ1	Actin, cytoplasmic 1	41.7	320.6	7
Q29108	Zona pellucida-binding protein	39.6	196.3	3
P36887	cAMP-dependent protein kinase catalytic subunit alpha	40.6	158	4
P62197	26S protease regulatory subunit 8	45.6	119.4	2
Q7YR83	Epididymal sperm-binding protein 1	26.2	112.8	3
P26776	Carbohydrate-binding protein AWN	16.7	107.8	2
Q710C4	Adenosylhomocysteinase	47.7	69.6	2
P24020	Carbohydrate-binding protein AQN-3	12.9	62.3	2
Band P56				
P08001	Acrosin	45.3	512.7	10
P02554	Tubulin beta chain	49.8	460.8	11
P79385	Lactadherin	45.7	443.5	12
Q29596	ATP synthase subunit alpha liver isoform, mitochondrial (Fragment)	15.6	272.2	5
Q29236	T-complex protein 1 subunit zeta (Fragment)	10.9	202.2	5
Q2XVP4	Tubulin alpha-1B chain	50.1	179.4	3

Table 4.5. *(continued)*

Accession[2]	Protein name[2]	MW (kDa)	Mascot score[2]	Peptides[3]
Band P56 continue				
Q9MYY8	Thioredoxin reductase 1, cytoplasmic	54.9	137.9	3
F1RKQ4	Bifunctional ATP-dependent dihydroxyacetone kinase/FAD-AMP lyase (cyclizing)	59.2	121.6	3
Q29068	T-complex protein 1 subunit gamma (Fragment)	8.7	121.1	2
P05207	cAMP-dependent protein kinase type II-alpha regulatory subunit (Fragment)	17.7	105.9	2
P79303	UTP--glucose-1-phosphate uridylyltransferase	56.9	86.7	2
Band P87				
O02705	Heat shock protein HSP 90-alpha	84.7	1380.3	25
P03974	Transitional endoplasmic reticulum ATPase	89.2	592.1	13
P16276	Aconitate hydratase, mitochondrial	85.7	486	8
Q2HYU2	6-phosphofructokinase, muscle type	85.3	341.7	7
P14632	Lactotransferrin	77.6	267.7	7
Q8SPR7	1-phosphatidylinositol 4,5-bisphosphate phosphodiesterase delta-4	88	258	7
P08001	Acrosin	45.3	216.9	5
P79385	Lactadherin	45.7	175.1	4
Q29554	Trifunctional enzyme subunit alpha, mitochondrial	83.1	155.8	5
Q29048	V-type proton ATPase catalytic subunit A	68.3	131	3
P26776	Carbohydrate-binding protein AWN	16.7	104.7	2
Q29092	Endoplasmin	92.4	94.4	2

Table 4.5. *(continued)*

Accession[2]	Protein name[2]	MW (kDa)	Mascot score[2]	Peptides[3]
Band P87 continue				
P36887	cAMP-dependent protein kinase catalytic subunit alpha	40.6	61.8	2
Q68J42	Hormone-sensitive lipase	83.4	61.8	2
Band P100				
Q1W674	Hexokinase-2	102.3	478.4	10
O02705	Heat shock protein HSP 90-alpha	84.7	477.1	9
P22411	Dipeptidyl peptidase 4	88.2	249.4	5
P08001	Acrosin	45.3	126.8	3
P36887	cAMP-dependent protein kinase catalytic subunit alpha	40.6	61.8	2
Q68J42	Hormone-sensitive lipase	83.4	61.8	2
P03974	Transitional endoplasmic reticulum ATPase	89.2	125.8	4
P19156	Potassium-transporting ATPase alpha chain 1	114.2	82.4	2
P79385	Lactadherin	45.7	79.6	2

[1]Proteins were purified by a sLe^X affinity column and subjected to SDS-PAGE. After protein separation, gel was stained with Coomassie Blue and protein bands were excised. Gel bands were trypsin digested and analyzed via LC-MS/MS on a Bruker amaZon ETD ion trap using CID for fragmentation. Data included proteins with Mascot score $\geq$ 50 and with at least 2 peptides.

[2] Data from UniProt databases. Database was searched using the Mascot search engine on the Matrix Science public domain server (www.matrixscience.com).

[3] Unique peptides identified.

Chapter 5. Identification of ADAM proteins as a Lewis X-binding receptor in epididymal boar sperm

Abstract

Attachment to oviduct glycan motifs including Lewis X structures (Le^X) is important for sperm retention by the oviduct epithelium and formation of a reservoir. The objective of this study was to purify relevant sLe^X-binding proteins that are involved in adhesion to the oviduct. To identify low abundant proteins that are usually masked by proteins originating from seminal fluid, sLe^X candidate receptors were purified from cauda epididymal boar sperm. To enrich for candidate receptors, plasma membrane preparations from cauda epididymal sperm were subjected to 3 separations steps prior to LC-MS/MS. By use of RP-HPLC and glycan blotting assays, it was possible to distinctively fractionate and identify multiple sLe^X receptors migrating at 11 to 60 kDa. Epididymal sperm-binding protein 1 and lactadherin were identified as proteins migrating at 25 kDa and 44 kDa. Both the unreviewed (B2CZF8) and reviewed (P79385) isoforms of lactadherin were identified. A band migrating at 44 kDa was identified as ADAM5 and it was associated with ADAM3a and ADAM2. In conclusion, the results described here confirm lactadherin as a sLe^X-binding protein. In addition, this study shows for the first time that ADAM5 binds the sLe^X motif, the first evidence that any ADAM binds carbohydrates. These results provide a mechanistic interpretation of the sperm transport phenotype observed in mice deficient in ADAM5 and other sperm ADAM family members.

1. Introduction

During movement through the oviduct, sperm are held in the lower region, known as the isthmus, where they form a reservoir and have a prolonged lifespan (Hunter 1984). It is believed that establishment of this reservoir involves adhesion of sperm lectin-like receptors to glycans on the surface of the oviduct epithelium (DeMott et al. 1995). We previously identified lactadherin and spermadhesin proteins from sperm as components that bound sulfated Lewis X and that were important for sperm binding to the oviduct (Chapter 4). Lactadherin, which is acquired during sperm transit through the epididymis, is a protein with EGF and discoidin domains and is involved in cell-cell adhesions (Petrunkina et al. 2003). Spermadhesins, the most abundant proteins in seminal fluid, coat the sperm surface after ejaculation (Topfer-Petersen et al. 1998). Both lactadherin and spermadhesin proteins on ejaculated sperm have been ascribed as mediators of the formation of the sperm reservoir in the pig oviduct (Ekhlasi-Hundrieser et al. 2005, Petrunkina et al. 2003). However, other proteins intrinsic to sperm are likely to have important roles in sperm oviduct formation.

The ability to bind oviduct epithelium gradually increases as sperm move from the epididymis to ejaculation (Petrunkina et al. 2001), indicating that proteins originating from accessory glands are necessary for achieving maximal binding ability. In bull sperm, addition of the seminal fluid protein PDC-190 to epididymal sperm increases sperm binding to oviduct cells to levels similar to ejaculated sperm (Gwathmey et al. 2003). Even if fewer cauda epididymal than ejaculated sperm bind to oviduct cells (Petrunkina et al. 2001), at least some of the sperm binding molecules have to be present before contact with the accessory gland fluid. Further, cauda epididymal sperm have normal fertility (Amann et al. 1993). Therefore, we hypothesized that sperm collected from the cauda epididymis would have biologically important oviduct

glycan receptors. It is known that epididymal sperm bind to sLe^X and SiLN motifs as efficiently as ejaculated sperm, indicating that the major protein/s involved in sperm-Le^X binding are present before ejaculation (Silva et al. 2013). Based on binding assays using fetuin and asialofetuin, distinct proteins mediate epididymal sperm binding to SiLN and Le^X motifs (Mori et al. 2000, Yoshitani et al. 2001). The use of epididymal sperm to purify sLe^X-binding proteins aims to identify low abundance proteins that have relevant biological function but may be masked by more abundant proteins originating from seminal fluid. In addition, the use of plasma membrane enriched samples is a more efficient approach to accurately identify proteins present on the sperm surface by reducing the presence of intracellular contaminants.

This study objectives are to identify candidate receptors for oviduct glycans using a stepwise approach that includes plasma membrane protein enrichment, reversed phase high performance liquid chromatography, SDS-PAGE and LC-MS/MS analysis. Results of this study will enhance the current understanding of how sperm recognize sLe^X and its potential role in sperm binding to oviduct epithelium cells. Identification of oviduct glycan receptors would also provide insight into how sperm lifespan is extended in the oviduct and would be targets for laboratory assays of fertility.

2. Material and methods

2.1. Materials

Chemicals were purchased from Sigma-Aldrich Co. (St. Louis, MO), unless stated otherwise. Carbo-free blocking solution was purchased from Vector Laboratory (Burlingame, CA). SuperSignal West Pico Chemiluminescent Substrate was purchased from Thermo Scientific

(Rockford, IL). Precast gel (4-20%) and Precision Plus Protein Standard Kaleidoscope Pre stained protein markers were purchased from Bio-RAD (Hercules, CA). Nonylphenyl Polyethylene Glycol (NP-40) was purchased from Calbiochem® EMD Millipore (Billerica, MA). Biotinylated sulfated Lewis X was donated from Lectinity (Moscow, Russia). BCA assay kit was purchased from Pierce Biotechnology (Rockford, IL).

2.2. Epididymal Sperm Collection and plasma membrane isolation

Boar testes were collected from a local abattoir and were used within 4 hours of slaughter. After arrival in the laboratory, the cauda epididymides were retrograde flushed with PBS (136 mM NaCl, 2.68 mM KCl, 10.4 mM Na_2HPO_4, 1.76 mM KH_2PO_4, pH 7.4) to remove sperm. Cauda epididymal sperm were separated from fluid by centrifugation (800xg for 6 min at 24°C). After centrifugation, cauda epididymal sperm were washed twice with Hepes Buffer Saline (HBS; 126 mM NaCl, 5 mM KCl, 18.2 mM HEPES, pH 7.4) at 800xg for 6 min, 24°C. HBS washed sperm were used instead of Percoll® washed sperm to preserve proteins loosely attached to the sperm surface (Russell et al. 1985). Sperm motility was determined and was > 70%. The enriched plasma membrane fraction was collected by nitrogen cavitation followed by a sequence of high speed centrifugation steps, as described (Flesch et al. 1998, Gillis et al. 1978). Briefly, washed ejaculated or cauda epididymal sperm were resuspended in Tris-Buffered sucrose solution (TBSS; 40 mM Tris Base, 50 mM sucrose and SigmaFAST^{TM} Protease Inhibitor Cocktail, pH 7.4). All subsequent media used during this procedure contained protease inhibitor. The sperm suspension ($1x10^9$/ml) was subjected to nitrogen cavitation (10 min, 50 bar) in a cell disruption bomb (Parr Instrument, Moline, IL). The cavitate was slowly released in a 50 ml tube, vortexed for 1 min and centrifuged at 1000xg for 15 min at 6°C. After centrifugation, the supernatant was collected. The pellet was washed with 3 ml of TBSS and centrifuged again at

1,000xg for 15 min at 6°C. This procedure was repeated. The supernatants were combined and centrifuged at 5956xg for 20 min at 6°C. The 5956xg supernatant was collected and subsequently centrifuged at 145,250xg for 60 min at 6°C to pellet the membrane proteins. The pellet was washed with HBS and centrifuged again at 145,250xg for 60 min at 6°C. After the last spin, the pellet was collected and solubilized with HBS + 0.1% NP-40 for 30 min in ice. The sample was centrifuged at 10,000xg for 2 min to remove any precipitate. The supernatant was collected and kept in -20°C until further analysis.

2.3. Reversed –phase high performance liquid chromatography (RP-HPLC)

After plasma membrane isolation, 2 mg of protein sample was fractionated with one step RP-HPLC. Reversed-phase HPLC was performed on a GE MDLC system. The column was a Vydac C4 (214TP54) 4.6 X 250 mm reversed-phase column run at 1 ml/min. The gradient was 10% to 90% acetonitrile in 0.1% TFA at 1 %/min. Fractions were collected and lyophilized for further use. Each fraction peak was resolved by one-dimensional SDS-PAGE followed by glycan blot analysis to investigate the presence of glycan binding proteins.

2.4. Glycan Blot

Each fraction with a distinct peak were analyzed for the presence of sLe^X-binding activity. Lyophilized protein fractions collected after RP-HPLC were resuspended in 46 µl of 4x Laemmli Buffer (40% glycerol, 200mM Tris (pH 8.0), 8% SDS, 400mM DTT, 0.1% bromophenol blue) diluted to 1x in PBS. Samples were heated at 95°C for 8 min and resolved by electrophoresis in a 4-20% gradient gel followed by transfer onto a nitrocellulose membrane. An aliquot of the sperm sample not submitted to RP-HPLC (input sample) was also included; 15 µg of unfractionated sperm membrane protein was loaded in the gel and transferred onto a

membrane. The membrane was blocked overnight at 4°C in blocking buffer consisting of 1x Carbo-free blocking buffer diluted in Tris Buffered Saline and Tween20 (TBST; 50 mM Tris Base, 150 mM NaCL, 0.1% Tween 20®, 0.2 mM $CaCl_2$ and 0.2 mM $MgCl_2$). After the blocking step, the membrane was washed 3 times for 10 min each with TBST and subsequently blotted with 1 µg/ml biotinylated sLe^X diluted in blocking buffer. After the glycan incubation and rinsing steps, the membrane was incubated for 30 min with Streptavidin Poly-HRP Conjugate (Pierce Biotechnology, Rockford, IL) diluted 1:60,000 in blocking buffer. Proteins that bound the biotinylated glycan were visualized after membrane incubation with ECL Western Blotting Substrate (Pierce Biotechnology, Rockford, IL). For a negative control, biotinylated glycan was omitted from the incubation.

Based on the glycan blot result, fractions containing sLe^X-binding proteins were B8, B9, B11, B12, C7, F2 and G4. These fractions were resolved in a second one-dimensional SDS PAGE and stained with SimplyBlue™ SafeStain (Life Technologies, Carlsbad, California) following manufacturer's instructions. sLe^X-binding proteins were identified based on their molecular weight. Bands were excised and submitted to LC-ESI-MS/MS.

2.5. LC-ESI-MS/MS

Gel samples were processed as described in Chapter 4. Samples were in-gel digested with trypsin plus ProteaseMax (Promega, Madison, WI) and then analyzed via LC-ESI-MS/MS using a top10 HCD MS/MS method. Data was processed using Q-Exactive acquisition data using Proteome Discover (Thermo Fisher Scientific, San Jose, CA). Mascot search algorithm (Matrix Science) was performed against the UniProt *Sus Scrofa* complete database (May, 2013) to identify proteins. Reported peptides were filtered to a false discovery rate of 1%. Reported proteins were filtered to require a Mascot score greater than 50 and at least two unique peptides

used for identification. Contaminant proteins such as keratins were not included in the reported identifications.

3. Results

3.1. Purification of sLe^X-binding proteins

As a first step to purify candidate sLe^X receptors from sperm, samples of enriched plasma membranes from cauda epididymal sperm were fractionated by reversed phase liquid chromatography (RP-HPLC), resolving a total of 19 distinct peaks (Figure 5.1). Fractions within each peak were pooled and resolved by SDS-PAGE. Proteins were transferred to nitrocellulose and the membranes were incubated with biotinylated sLe^X to detect bands with affinity for this trisaccharide. Two sLe^X-binding proteins migrating at 44 kDa were detected in fractions B8 and B9, and proteins migrating at 60 kDa were detected in fractions F2 and G4. Other proteins migrating at 11, 25 and 47 kDa were detected in B11, B12 and C7, respectively. Each protein band was identified as protein sample (PS) 1-7 as illustrated in Figure 5.2A. Five of the proteins identified as sLe^X-binding protein after RP-HPLC were detected in the input sample, whereas PS3 and PS5 were not detected (Figure 5.2B). Analysis of the input sample revealed a 35 kDa protein that was not detected after RP-HPLC. In the negative control blot, a nonspecific signal was detected in fraction B8 at the same location of PS1.

All fractions containing a sLe^X-binding protein were subjected to preparative SDS-PAGE before LC-MS/MS. After SDS-PAGE, the gel was stained with SimplyBlue™ SafeStain to locate proteins within each fraction that bound glycans. PS1 and PS2 were identified as a double band migrating at 44 and 38 kDa (Figure 5.2C). Based on the glycan blot result illustrated in

Figure 5.2A, only the higher MW protein bound to the biotinylated sLe^X. The lower MW protein was identified as PS1* and PS2* and were submitted to LC-MS/MS to further investigate a possible complex formation with PS1 and PS2.

In fraction B11, PS3 appears to be a double band with a faint signal of 12 kDa. As expected, two bands appeared as PS3; the higher MW protein was identified as PS3*. Both PS4 and PS5 appeared as a single band whereas PS6 and PS7 were not detected in the Coomassie Blue stained gel.

3.2. Identification of sLe^X binding protein by LC-MS/MS

All the protein bands detected by a Coomassie Blue stain were excised from the gel and submitted to LC-MS/MS. Searching an unreviewed database for identification of peptide sequences revealed multiple proteins with Mascot scores > 10,000 (Table 5.1).

PS1, PS1*, PS2 and PS2*

Database search identified both PS1 and PS2 as disintegrin and metalloprotease domain-containing protein 5 (ADAM5; accession number E7FM66) which has a MW of 45.2 kDa (Uniprot Database). ADAM5 was identified with high confidence based on a Mascot score of 23587.6 and 6046.0 for PS1 and PS2, respectively. A high percentage of the protein sequence was covered (50 and 47.3% for PS1 and PS2, respectively). The peptides identified within PS2 are highlighted in Figure 5.3. PS1* and PS2* were both identified as ADAM3a and ADAM2. The molecular weights of ADAM3a (82.9) and ADAM2 (81.8 kDa) do not match with the 38 kDa estimated molecular weight of the gel bands submitted to LC-MS/MS. However, investigation of the peptide sequences identified by MS/MS demonstrated that only the C

terminal half of the protein sequence was present for both ADAM3a and ADAM2, explaining the migration as 38 kDa protein (Figure 5.4).

PS3 and PS3*

Database search for the identity of PS3 did not result in relevant candidates. The proteins with highest Mascot scores were lactadherin and epididymal sperm binding protein 1. Additionally, database search for PS3* identified the unreviewed protein LOC100625848 and the intracellular protein peptidyl-prolyl cis-trans isomerase. There is no evidence that either protein contains domains that may bind glycans.

PS4

PS4 was confidently identified as epididymal sperm-binding protein 1 which has a molecular weight of 26.2 kDa, similar to the molecular weight of the gel band excised for MS/MS. It had a very high Mascot score of 29488 and 68.2% of the protein was covered by sequences identified by MS/MS.

PS5

The protein corresponding to PS5 is most likely lactadherin. Both the unreviewed (accession: B2CZF8) and reviewed (accession: P79385) isoforms were identified. Peptide sequences deduced by LC-MS/MS for both proteins included the discoidin domain (data not shown).

4. Discussion

Glycan-binding proteins were efficiently isolated from epididymal sperm plasma membrane proteins by reversed phase high performance liquid chromatography and one-dimensional SDS-PAGE. With the exception of PS3*, all the protein identified (ADAM5, epididymal sperm binding protein 1 and lactadherin) are sperm surface proteins. Use of nitrogen cavitation and the centrifugation steps isolates plasma membrane proteins with high purity (Flesch et al. 1998). In addition, RP-HPLC was an efficient approach to fractionate epididymal proteins with high resolution, resulting in detection of two sLe^X-binding proteins (PS3 and PS5) that were not detected in analysis of unfractionated sample.

The sLe^X-binding proteins PS1 and PS2 were identified as ADAM5. By glycan blotting, PS1 elicited a strong nonspecific signal that we speculate may be due to the high amount of protein loaded in the gel. The same protein, PS2, did not elicit a strong nonspecific signal and supports the conclusion that ADAM5 is a sLe^X-binding protein. The *a d*isintegrin *a*nd *m*etalloprotease (ADAMs) family is large, containing at least 33 members, 18 of which are present in the male reproductive system (Han et al. 2009). ADAM proteins have both adhesion and proteolysis function due to a multidomain structure characterized by a metalloprotease domain as well as a disintegrin domain (Wolfsberg et al. 1995b). ADAMs proteins contain a disintegrin domain that recognizes integrins present on the oocyte zona pellucida promoting sperm binding (Chen et al. 1999). The disintegrin-like domain mediates cell adhesion; however, not all ADAM proteins are functional adhesion molecules (Wolfsberg et al. 1995a). Using an asialo-α1-acid and asialo-agalacto- α1-acid affinity chromatography, it has been reported that *N*-acetyllactosamine is the ligand for ADAM5 in epididymal boar sperm but not Le^X (Mori et al. 2012). Boar epididymal and ejaculated sperm recognize *N*-acetyllactosamine but with

significantly lower binding than to Le^X (Silva et al. 2013). Results described here support that oviduct sLe^X is a ligand for sperm ADAM5.

PS1* and PS2* were identified as ADAM2 and ADAM3a, which are present as the 40-45 kDa mature form. ADAM proteins are also detected with a higher molecular weight that corresponds to the precursor protein that is processed to mature form in the epididymis (Kim et al. 2006). ADAM2, ADAM3 and ADAM5 are present on the mouse sperm surface and are part of the same ADAM phylogenetic group characterized by proteins present exclusively or predominantly in the testis (Kim et al. 2006). ADAM2 and ADAM3 regulate ADAM5 abundance on the sperm surface (Kim et al. 2006). It will be very interesting to determine if the proteins ADAM3a, 2 and 5 form a protein complex to recognize Le^X. In mice, it has been speculated that ADAM5 is downstream of ADAM1a/2 complex and that these ADAMs play a coordinated role in sperm migration to the oviduct (Kim et al. 2006).

The proteins identified as PS4 and PS5 were epididymal sperm binding protein 1 and lactadherin. Previous data using ejaculated sperm has determined that both proteins bind sLe^X and lactadherin is involved in sperm recognition of oviduct epithelial cells (Chapter 4). The results using epididymal sperm in the present study corroborate the interaction between lactadherin and sLe^X and highlight the importance of these co-receptors as adhesion molecules. Similar to ADAMs proteins, lactadherin is also known to bind integrins (Raymond and Shur 2009) but it is unknown if this interaction is through lactadherin recognition of integrin carbohydrates.

In summary, glycan-binding proteins were efficiently isolated from boar epididymal sperm membranes using reversed phase liquid chromatography and one-dimensional SDS-

PAGE. Data presented here support the hypothesis that multiple proteins coordinate binding to sLe^X and potentially mediate sperm adhesion in epididymal sperm. We have purified and identified ADAM5, lactadherin and ESBP1 as sLe^X-binding proteins. Lactadherin has been described as a receptor to sLe^X but this is the first time that ADAM5 has been described as a sLe^X-binding protein. Because ADAM5 contains a transmembrane domain, it is able to induce an intracellular response through stimulation of signal pathways, something less likely with peripheral membrane proteins such as lactadherin or ESBP1. It is recognized that there is cross-talk between the sperm and oviduct cells that influences oviduct cell gene expression and protein secretion as well sperm function (Fazeli et al. 2004, Georgiou et al. 2005). In addition, the interaction of sperm with glycans can affect sperm function. For example, Le^X in uterine fluid influences mouse sperm motility (Kuo et al. 2009); therefore, it is likely that Le^X in the oviduct epithelium may also impact porcine sperm function. Determination of molecules capable of eliciting a cell signaling response is of great importance for further understanding of how sperm adhesion to glycans can impact sperm physiology. It is possible that ADAM5 protein present in the sperm plasma membrane produces a specific response once bound to the oviduct epithelium maintaining the sperm in a quiescent state. We are still in the beginning stages of identifying and characterizing the glycan receptors and the mechanisms of signal transduction in the sperm.

Acknowledgments

I would like to thank Dr Peter Yau and Dr Brian Imai for their assistance with the protein purification process and valuable discussions.

FIGURES AND TABLES

FIGURE 5.1.

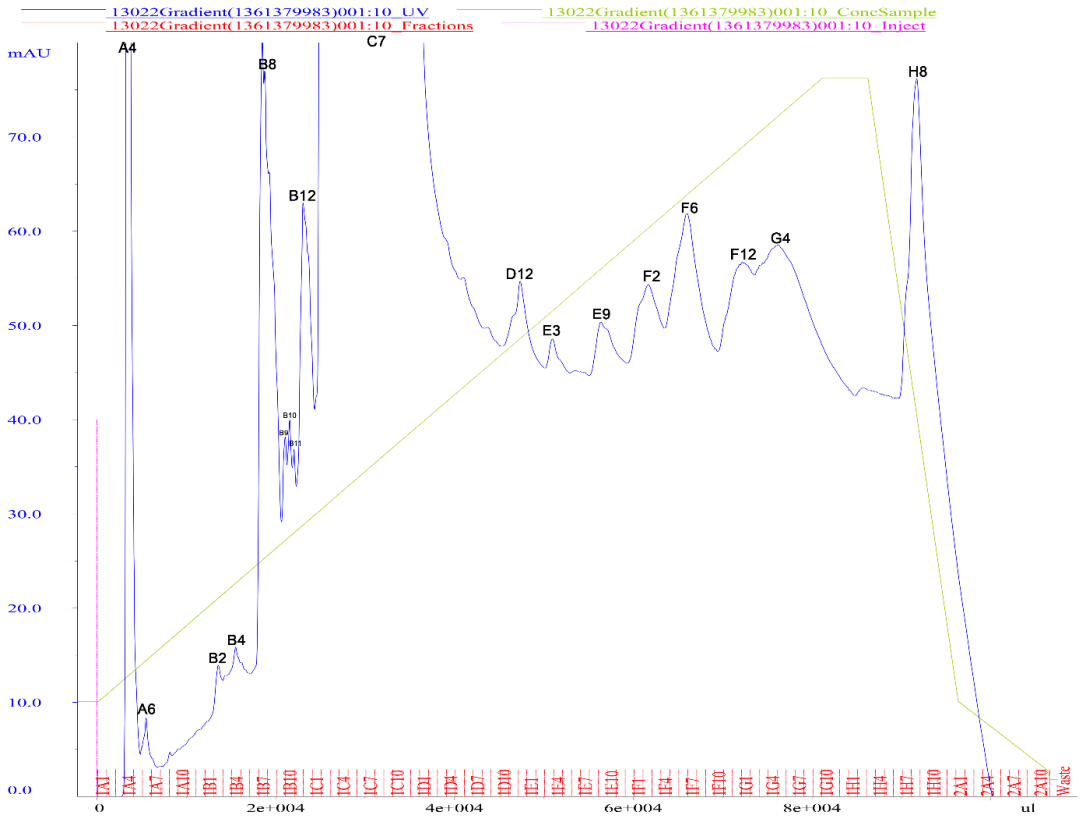

Figure 5.1. Fractionation of sperm proteins by reversed phase HPLC (RP-HPLC). Proteins isolated from epididymal sperm plasma membranes were applied to a C18 HPLC column and eluted with an increasing gradient of acetonitrile. A total of 19 peaks containing protein were identified for further analysis.

FIGURE 5.2.

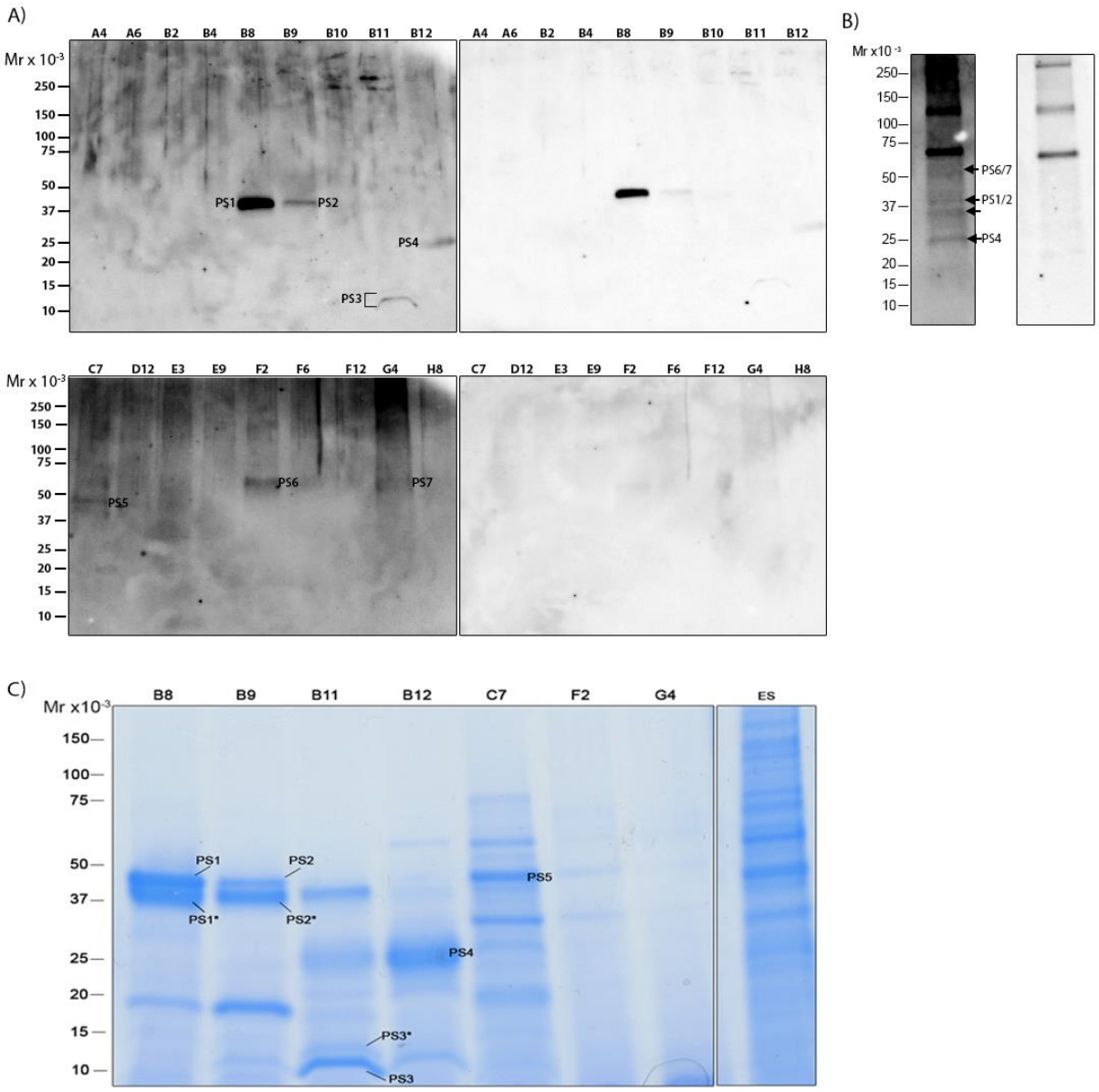

Figure 5.2. Detection of sLe^X-binding proteins in RP-HPLC fractions. A) Glycan blotting identified sLe^X- binding proteins in fractions B8, B9, B11, B12, C7, F2 and G4. Each band was identified as protein sample (PS) 1 -7. Negative control blots, where biotinylated glycan was omitted, are shown on the right. A nonspecific band was present in fraction B8. B) Glycan blotting assay of the input sample shows that sLe^X-binding proteins migrated at sizes ranging from 25 to 70 kDa (black arrows). Negative control is shown on the right. The migration matched with migration of PS1/ PS2, PS4 and PS6/7. C) RP-HPLC fractions containing sLe^X-binding proteins were further resolved by one-dimensional SDS PAGE and gel was stained with Coomassie Blue. Proteins were identified based on their molecular weight. PS1 and PS2 appear as double bands; lower MW proteins were identified as PS1*and PS2*. PS6 and PS7 were not detected in fractions F2 and G4. The starting material, the epididymal sperm (ES) plasma membrane sample, is shown on the right.

Table 5.1. LC-MS/MS identification of sLe^X-binding proteins[1].

Protein name	General name	Accession	Mascot score	MW (kDa)	Coverage (%)	Peptides	Unique Peptides
Protein sample1 (PS1)							
Disintegrin and metalloprotease domain-containing protein 5	ADAM5	E7FM66	23587.6	45.2	50.0	27	27
ADAM3a	-	A5A4F6	7090.4	82.9	18.4	16	16
Fertilin beta	FTNB	Q866A8	5487.8	81.8	13.7	11	11
Protein sample2 (PS2)							
Disintegrin and metalloprotease domain-containing protein 5	ADAM5	E7FM66	6046.0	45.2	47.3	19	19
ADAM3a	-	A5A4F6	3839.3	82.9	14.8	13	13
Protein sample1* (PS1*)							
Fertilin beta	FTNB	Q866A8	38493.9	81.8	15.9	14	13
ADAM3a	-	A5A4F6	23336.8	82.9	23.4	19	19
Disintegrin and metalloprotease domain-containing protein 5	ADAM5	E7FM66	11912.1	45.2	48.5	24	23
Protein sample2* (PS2*)							
ADAM3a	-	A5A4F6	17509.7	82.9	22.5	17	17
Fertilin beta	FTNB	Q866A8	8947.4	81.8	14.4	11	10
Uncharacterized protein	-	F1RZL9	4119.2	83.8	19.2	17	2
ADAM3b	-	A5HJZ3	3932.8	83.5	22.4	17	2

Table 5.1. *(continued)*

Protein name	General name	Accession	Mascot score	MW (kDa)	Coverage (%)	Peptides	Unique Peptides
Protein sample 3							
Lactadherin	MFGE8	B2CZF8	1386.7	47.8	34.1	15	11
Epididymal sperm-binding protein 1 (reviewed protein)	ELSPBP1	F1RLA2	839.3	26.2	38.6	9	9
Protein sample 3* (PS3*)							
LOC100625848	-	I3LPE2	2509.4	18.9	67.2	13	13
Peptidyl-prolyl cis-trans isomerase	FKBP2	F1RQ06	1885.4	15.4	40.0	10	9
Protein sample 4 (PS4)							
Epididymal sperm-binding protein 1 (reviewed protein)	ELSPBP1	F1RLA2	29488.0	26.2	68.2	14	14
Protein sample 5 (PS5)							
Lactadherin	MFGE8	B2CZF8	19511.2	47.8	62.9	37	6
Lactadherin (reviewed protein)	MFGE8	P79385	18694.5	45.7	61.6	32	1

[1]Proteins from epididymal sperm plasma membrane were identified by MS/MS. Proteins were excised from a Coomassie stained one-dimensional gel. Database was searched using the Mascot engine on the Matrix Science public domain server (www.matrixscience.com).

Table 5.2. Peptide sequences from PS2 identified as ADAM5 by MS/MS analysis.

Number	Peptides Identified	Ion score	# PSMs	MH+ (Da)
1[a]	EQmcVSTFLNAEKIPR	100.0	13.0	1938.94158
2	NGQScESGSAYcYGGR	89.0	13.0	1752.66655
3	DTITTVQFPGDR	85.6	10.0	1349.66899
4	IcGNGILEQGEQcDcGTLENcTHK	82.0	1.0	2793.17887
5	NLIGGESTGASFScFDEINSRK	81.2	1.0	2389.10758
6	QcGSGEccTQDcK	77.8	6.0	1589.54436
7	KGFVPPDcNVGNGFGSIDDGHQSK	70.2	16.0	2532.16220
8[b]	EQmcVSTFLNAEK	68.8	23.0	1572.70513
9[b]	EQMcVSTFLNAEK	65.8	4.0	1556.70928
10	EYcNYPHLLcGK	65.4	19.0	1553.68572
11	HcNANGVcNNFNHcHcK	64.2	2.0	2142.82523
12	GFVPPDcNVGNGFGSIDDGHQSK	50.8	26.0	2404.06217
13	LVcNWPHK	49.8	4.0	1053.53105
14	DTITTVQFPGDRDR	46.8	39.0	1620.80168
15[a]	EQMcVSTFLNAEKIPR	45.5	2.0	1922.95237
16	IRPANVIcR	37.1	11.0	1098.62261
17	FGNcGR	24.0	9.0	710.30309
18	HccDPR	21.5	11.0	844.31963
19	QcGSGEccTQDcKIRPANVIcR	20.2	1.0	2669.16396
20	NKQcGSGEccTQDcK	16.9	2.0	1831.68308
21	DRFGNcGR	14.9	1.0	981.43151

[ab] Similar superscripts indicate that the same peptide sequence was identified.

FIGURE 5.3.

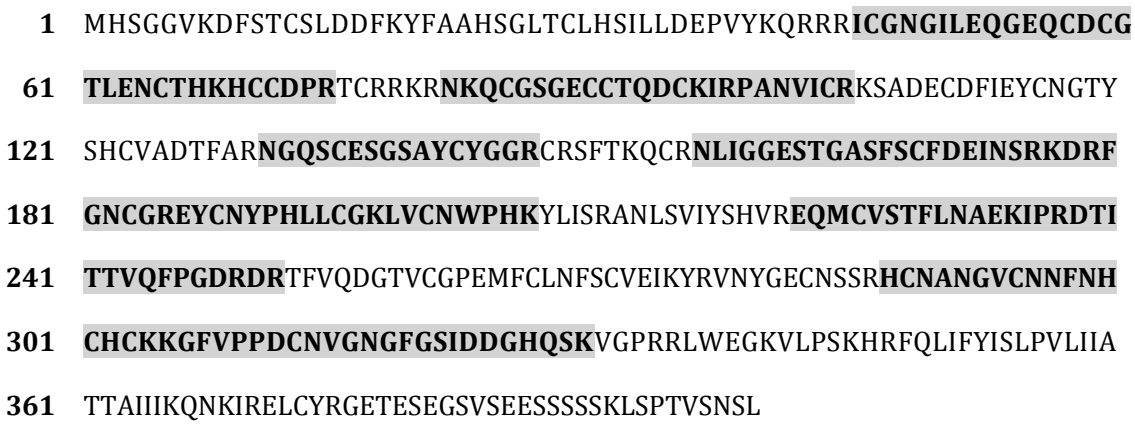

Figure 5.3. Amino acid sequence of the unreviewed protein ADAM5 (accession number E7FM66). Highlighted peptide sequences were deduced by LC-MS/MS.

FIGURE 5.4.

ADAM3a

1 MLPFLLLLSGLSQLTSAGHHSETSLLQITVPQKIGTNTKDSGASDTNVTYDIKINRKTFT
61 LHLEKQSFLDSNFLVYSYNKSGVLYPDSSFIKGHCFYQGYAGEIPNSVVTLSTCSGLRGL
121 LQFENISYGTEPLESSATYEHIIYQIKNNKSDFSPIARNYSTTQFAAQPFKILVKSEKKS
181 EVLLKRILKIQVIMDKALYDYMGSEVALASEKIVYVFSLINNMFSQLKMTVMLTSLELWS
241 DKNQILTNGDANEMLQKFVSWKEKKLFQRSHDMAYLLIYRDHPNYIGATYHGMACNPKFA
301 AGIALYPKMITLEAFSVILAQLIGISLGLTYKIDIYNCYCPGTICIMNPEAIRSRGVKFF
361 SSCSVDEFKSTVSRPEFECLQNQIVPKVVPQAR**TGSCGNGAMEEGEQCDCGAVENCIHK**K
421 **CCDAANCTLIGDAECGTGPCCDRK**TCRLSQRGVLCR**RSTDLCDFPEYCSGTSEFCTEDMR**
481 AADFEMCNNK**TTYCFQGICRDRSKQCLELFGK**FAESSSYLCAQEVNIQSDIFGNCDGR**SC**
541 **NFVNTLCAKLVCHWKHSQIVPKTDFDIQYTYLDGHVCMSGNLR**NSSTPDLTK**VWDGTKCD**
601 **DGRFCDNGQCRLTQDYR**NRPNCSSAVKCGGHGLCNTLLNCHCDVGYAPPACEPEPLSPGG
661 SINDGFWIVEGKNVEFLVKRHRASQKKGLLISFYVLLPLLVLIAIVALQWNKKKIFWNRE
721 DTVSEGSPSEASSSKSDLSCSKSD

ADAM2

1 MLCLLFLLIGFLGLQADDSSERLRVQITVPEKIRSISSEGVESHVSYNIIIEGKTYTVNL
61 VQKSFLPHNFRVYGYNGTGSMKPLEQQFQNFCYYQGSIEGYPNSMVIVSTCTGLRGLLQF
121 ENVSYGIEPLEPSVGFEHVIYQVKHRSAGSSLYAEKDTESREMHYKIQSIEPLTGFIRYI
181 EIHIVVEKNLYNHMGSDTAVVTQKIFQLIGLTNAIFTSFNITIILSSLELWIDENKITVT
241 GDANELLHRFLKWKRSYLVLRPHDVAYLLVYREKPNFVGATFQGKICDKQYGGGIVLHPK
301 TITLESLAVTMAQLLSLSMGIAYDDINKCHCPGNVCIMNPEAIHSSGVKFFSNCSMEDFA
361 HFTAKSKSQCLQNQPRVDPSYKSAVCGNGEVEEGEECDCGTPENCAAQPNACCNQATCTL
421 SAGSACATGPCCDSCSFMAKGQTCRLTLDECDLLEYCNGSSAACQEDLYVQDGHPCSDNQ
481 WLCVQGKCISGMK**QCSETFGDGANYGTEDCFTHLNSKTDESGACGLTASGYIRCHPNDLR**
541 CGKIICTYEGTTILTIPNATITYSNINGK**ICVTVDYPYNDRNSEKMWVKDGTVCGENKVC**
601 **R**SK**RCIDSSYLNYDCTAEKCHNQGVCNNKR**NCHCKPTYLPPNCEVSVESWPGGSVDSGNF
661 PPAADPLPGVPDRRYIENIYHSRPMKWPFFLLIPFFIILCVLIATLVKIYFQRKKGRTED
721 DTSDEQLESESETKE

Figure 5.4. Amino acid sequence of the unreviewed protein ADAM3a (accession number A5A4F6) and ADAM2 (accession number Q866A8). Highlighted peptide sequences were deduced by LC-MS/MS.

Chapter 6. Conclusions and future direction

In this PhD dissertation, the first specific aim was to detect and characterize receptors for sLe^X and SiLN motifs that are present on the surface of epididymal and ejaculated pig sperm. The main findings from Chapter 3 are:

1) Multiple proteins recognize sLe^X as well SiLN motifs. With the use of fluorescent binding assays, I could determine differences in binding sites for each of the glycan investigated indicating the presence of distinct receptors. Even if the identity of these molecules were not initially determined, based on their migration pattern in a glycan blot it was hypothesized that the same protein could recognize different glycan motifs.

2) Specific binding to these motifs require divalent cations. In the presence of EDTA, a divalent cation chelator, sperm binding to fluorescent probes were reduced to levels similar to LN, our negative control, indicating that receptors of importance are characteristics of C-type lectins.

3) Glycan-binding proteins are present before sperm contact with accessory gland fluids. Investigation of epididymal sperm capacity to bind sLe^X and SiLN motifs determined that some sperm adhesive molecules are already present before ejaculation.

The information described in Chapter 3 predicted characteristics of an authentic sLe^X and SiLN receptor and was further applied in the design of the experiments illustrated in Chapter 4 and 5. In Chapter 4, sulfated Lewis X affinity chromatography was successfully applied to identify a cohort of glycan receptors in ejaculated sperm. Purification of multiple sLe^X receptors provided support to our previous theory that a complex system mediates sperm adhesion to oviduct cells. Lactadherin, a protein largely known to be involved in cell-cell adhesion systems, was purified and further validated as a sperm adhesive molecule involved in oviduct cell binding.

Because the ejaculated sperm surface is covered with proteins originating from accessory gland fluid, it is a challenge to identify low abundant proteins which may have significant biological function. To address this issue, in Chapter 5, I chose to use epididymal sperm for the protein purification procedure and a 44 kDa protein was isolated and further identified as ADAM5. This protein was present in the same fraction as ADAM3a and ADAM2 indicating that these proteins maybe form a complex that coordinates sperm binding to the oviduct epithelium.

In conclusion, the results described in this PhD dissertation have added novel information about how the sperm recognize carbohydrate structures. Lactadherin was purified through two different methodologies corroborating this protein as a sLe^X candidate receptor. In addition, this was the first time that ADAM5 affinity to sLe^X motif was demonstrated and this finding has a profound effect on the current knowledge regarding sperm interaction with the oviduct. Based on these discoveries, I propose a novel adhesion model that includes multiple proteins that work in a collective manner to promote sperm attachment to the oviduct epithelium (Figure 6.1). This adhesion model and the information generated in this PhD dissertation can also be applied to a better understanding of other cell-cell adhesion systems.

For future studies, there are several questions that need further investigation to support the results of this PhD dissertation. Such experiments are designed to further clarify the association of multiple proteins in sperm adhesion and also the possibility that a similar cell-cell binding model is present in other reproductive systems. In addition, as discussed in the introductory section of this dissertation, sperm bind with great affinity to sialylated *N*-acetyllactosamine; however, I have not identified a specific receptor for this glycan motif.

1. Do lactadherin and ADAM5 cooperate to coordinate pig sperm adhesion to the oviduct epithelium?

Distinct sperm proteins cooperate to adhere to zona pellucida carbohydrate motifs (Thaler and Cardullo 1996); therefore, it is possible that a similar system coordinates adhesion to the oviduct epithelium. To address this question I am planning to use imunoprecipitation assays to isolate proteins that are associated with lactadherin. Initially, I am going to use mouse lactadherin antibody, since this antibody recognizes pig lactadherin protein. My working hypothesis is that this protein is associated with ADAM5 and coordinates a secondary binding site as described for zona pellucida binding (Ensslin M. A. and Shur 2003). The next step is to produce pig ADAM5 antibodies to perform similar type of experiments and clarify the involvement of ADAM3 and ADAM2, as well other ADAM proteins that might function in this complex.

2. Does ADAM5 recognize sulfated Lewis X motif?

Even if I identified ADAM5 as a sLe^X receptor based on glycan blot results, further experiments are necessary to validate this result. For all *in vitro* studies, multiple experiments are necessary to accurately corroborate a scientific finding. Because there is no pig ADAM5 antibody or recombinant protein commercially available, validation of ADAM5 as a true sLe^X receptor was restricted in the current work. In addition, since our ultimate research interest is the establishment of the oviduct reservoir, I have to confirm the presence of this protein in ejaculated pig sperm instead of just epididymal sperm.

A limitation of the work with ADAMs proteins are that these proteins are usually active as dimers or associated with other proteins. It is necessary to investigate which components are

required for ADAM5 protein to bind sLe^X or SiLN structures, or even for the protein to be functionally active.

3. Does lactadherin play a role in zona pellucida interaction?

Because lactadherin is associated with a variety of cellular interactions, it is worthy to investigate lactadherin involved in pig sperm bind to oocyte zona pellucida. Even if it has established that proteins involved in sperm attachment to oviduct cells are lost after capacitation, results described in this dissertation can be translated into the sperm-oocyte binding model since both lactadherin and ADAM5 have been proposed as mediators of sperm adhesion to the zona pellucida (Ensslin M. A. and Shur 2003, Mori et al. 2012).

4. Are lactadherin or ADAM5 involved in SiLN binding?

Based on results described in Chapter 3, it was proposed that the same glycan-binding protein could bind both sLe^X and SiLN termini. During my research experiments, I focused on identifying sLe^X-binding proteins in an attempt to also identify a true SiLN-binding protein. Whether lactadherin or ADAM5 also bind SiLN remains to be determined. In addition, I cannot rule out the possibility that sLe^X receptors are actually recognizing complex carbohydrates with both the Le^X and SiLN motifs (glycan structure illustrated in Chapter 2, Figure 2.2). For instance, it is proposed that lactadherin has affinity for negative charged glycans, including those with sialic acid residues of SiLN.

Further studies are necessary to investigate if lactadherin also has specificity for the SiLN motif. The latter can be achieved by experiments using recombinant protein since biotinylated

SiLN reagent is available. In addition, an affinity purification process specifically targeting SiLN receptors may discover unique proteins not described in this dissertation.

FIGURES

FIGURE 6.1.

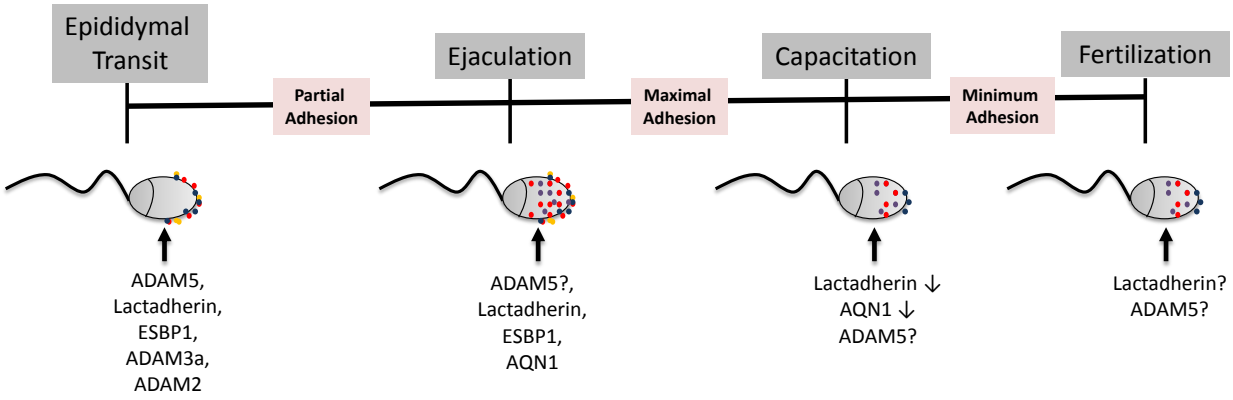

Figure 6.1. Proposed sperm adhesion model to the sLe^X motif present on the oviduct epithelium. ADAM5, lactadherin and ESBP1 were identified as sLe^X candidate receptors in epididymal sperm (Chapter 5) and may account for the partial epididymal binding to the oviduct epithelium. After ejaculation, when maximal sperm binding is achieved, AQN1 is attached to the plasma membrane and contributes to binding (Ekhlasi-Hundrieser et al. 2005). Lactadherin, still present on the sperm surface, also mediates this interaction (Chapter 4). After capacitation, AQN1 is lost from the membrane and account for the reduced sperm capacity to bind oviduct cells (Ekhlasi-Hundrieser et al. 2005). The presence of lactadherin and ADAM5 on the sperm surface after capacitation and their potential role in oocyte binding are under investigation.

Appendix A. The impact of altered leptin and glucose concentrations on ovarian cells

1. Introduction

Despite the fact that it has been well characterized in human and animal models that excess energy balance negatively affects fertility, the reasons about how these two systems interact still largely unknown. The oocyte, cumulus cells, granulosa and theca cells are in reality a functional unit, with dynamic communication occurring between each cell type (Kotsuji et al. 1990). The action of the adipokine leptin in each cell type is important for the correct function of the ovary, and consequently acquisition of oocyte competence followed by successful fertility (Paula-Lopes et al. 2007). However, it is not well understood how the alteration on glucose concentration would affects leptin role on oocyte function.

Leptin is the best studied adipokine; its serum concentration is positively correlated with fat mass since it is produced primarily by the adipocyte and its primary function is to control food intake and energy metabolism (Halaas et al. 1995). Leptin has been shown to have reproductive effects; homozygous mutation of the leptin gene (ob/ob) in a female mouse model leads to infertility, which can be reversed with leptin administration even before weight loss occurs, demonstrating the importance of leptin for normal reproductive function (Chehab et al. 1996). Despite the many reports describing the link between leptin and fertility, the pathways of leptin action in the ovary are not well understood.

Leptin receptor expression has been detected in the ovary in several species, including human, pig and mouse (Cioffi et al. 1997, Ruiz-Cortes et al. 2000, Ryan et al. 2002). In a study with transfected cells, both long and short forms of the leptin receptor can mediate tyrosine phosphorylation of IRS-1, JAK-2 and the MAPK pathway, but only the full-length form contains

the intracellular domains necessary to mediate activation of STAT3 tyrosine phosphorylation (Bjorbaek et al. 1997). Others have reported that the short form (OBRa) is able to initiates signal transmission (Murakami et al. 1997). In the ovary, both long and short forms of the receptor are present in human theca and granulosa cells, but the short form is expressed at a higher level (Karlsson et al. 1997). Similarly, low expression of OBRl was detected in porcine oocytes (Craig et al. 2004).

One possible link between leptin and reproduction is insulin, as obesity is associated with altered insulin signaling in non-reproductive tissues. Insulin binding to its receptor initiates phosphorylation of insulin receptor substrate 1 and 2 (IRS 1/2) resulting in a cascade of events including phosphorylation of phosphatidylinositol 3-kinase (PI3K) and activation of the RAS-MAP kinase pathway (Ogawa et al. 1998). PI3K is a major regulator of glucose transport and uptake, and MAPK activation is related to cell growth and DNA synthesis. Insulin receptor is present in pig and mouse oocytes (Acevedo et al. 2007, Quesnel 1999). Insulin individually or associated with LH and FSH regulates granulosa and theca cell steroidogenesis (Diamanti-Kandarakis and Papavassiliou 2006). The mechanism of insulin control of ovarian steroidogenesis is controversial, mainly because of post binding receptor divergences (Poretsky 1991). In addition, the type of cell and stage of follicular growth potentially induces different signals. Obese women have altered levels of circulating steroids (Azziz 1989) and defective insulin signaling has been proposed as the cause of changed ovarian function

Studies have focused on phosphorylation of the IRS proteins by agents as fatty acid, tumor necrosis factor α and cytokines as a mechanism that terminates insulin signal transduction and causes insulin resistance in peripheral tissue (Zick 2001). Leptin is known to phosphorylate ser 318 in IRS-1 in vitro and in obese individuals (Hennige et al., 2006). In cultured hepatic

cells, leptin modulates insulin action through dephosphorylation of IRS-1 and disruption of insulin signaling (Cohen et al. 1996). In the ovary, unspecific IRS-1 phosphorylation would result in altered ovarian steroidogenesis. Thus, it is possible that leptin may alter IRS phosphorylation to affect insulin control of ovarian steroidogenesis in obese individuals as highlighted in Figure A.1.

When conditions of excess energy occur, alterations in concentrations of glucose, leptin and triglycerides are thought to reduce fertility by acting upon the cells within the ovary to alter follicular growth, hormone production and oocyte development. Presence of leptin in a high glucose environment increases the number of oocytes arrested in germinal vesicle stage (Figure A.2), demonstrating the interaction between these two components. However, the cellular mechanisms by which these changes occur are unclear. The proposed specific aims will clarify the cellular signaling mechanisms within the ovary by which leptin and glucose work to impact female reproduction.

Specific Aim 1. *Evaluate alterations of oocyte function due to varying concentrations of leptin and glucose.* The working hypothesis of Specific Aim 1 is that leptin's effect on porcine oocytes is dependent on glucose conditions. Results from Specific Aim 1 are described elsewhere (Silva et al. 2012).

Specific Aim 2. *Determine the impact of the adipokine leptin on theca cells steroidogenesis in vitro.* The working hypothesis of Specific Aim 2, which is part of Appendix B, is that theca cells have altered insulin signaling pathways in response to altered leptin concentrations that subsequently affect steroidogenesis.

Specific Aim 3. *Investigate alterations in oocyte developmental competence due to alteration in lipids metabolism.* The working hypothesis of Specific Aim 3, which is part of Appendix C, is

that treatment of oocytes with an inhibitor of beta oxidation will impact oocyte function and nuclear maturation.

Our ultimate goal is to understand the impact of metabolic alterations in the follicle and how oocyte quality is altered in differing physiological conditions. The knowledge acquired by the proposed Specific Aims will increase our understating of how different molecules interact and potentially impact ovarian cells function.

FIGURES

FIGURE A.1.

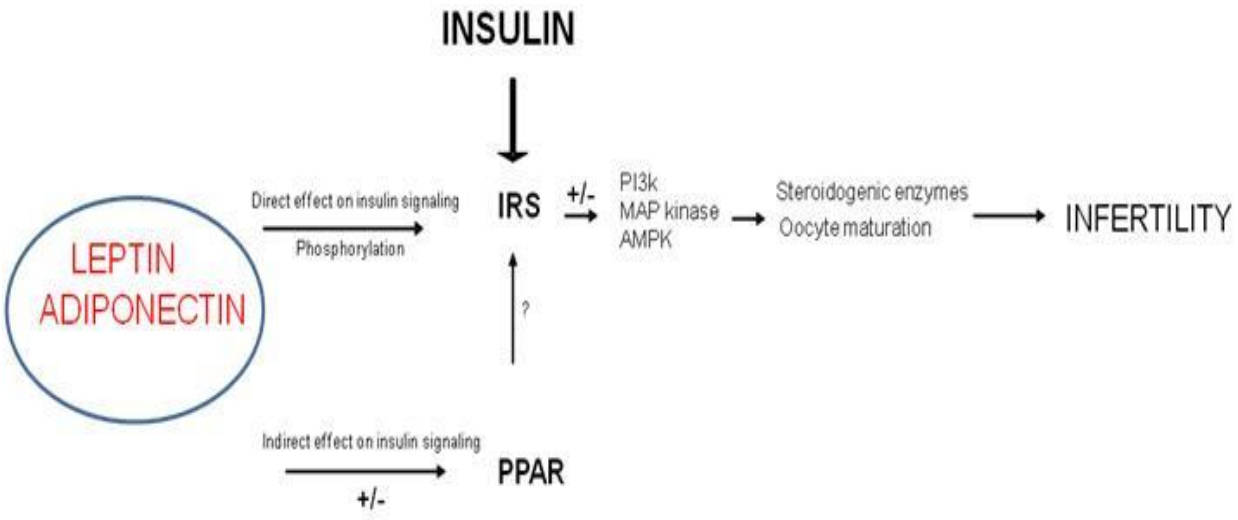

Figure A.1. The adipokines leptin and adiponectin alter IRS phosphorylation interfering with the insulin signaling pathway consequently affecting fertility. It is also hypothesized that these adipokines have an indirect effect on IRS through PPAR regulation; however, how PPAR participates in the insulin pathway is still unclear.

FIGURE A.2.

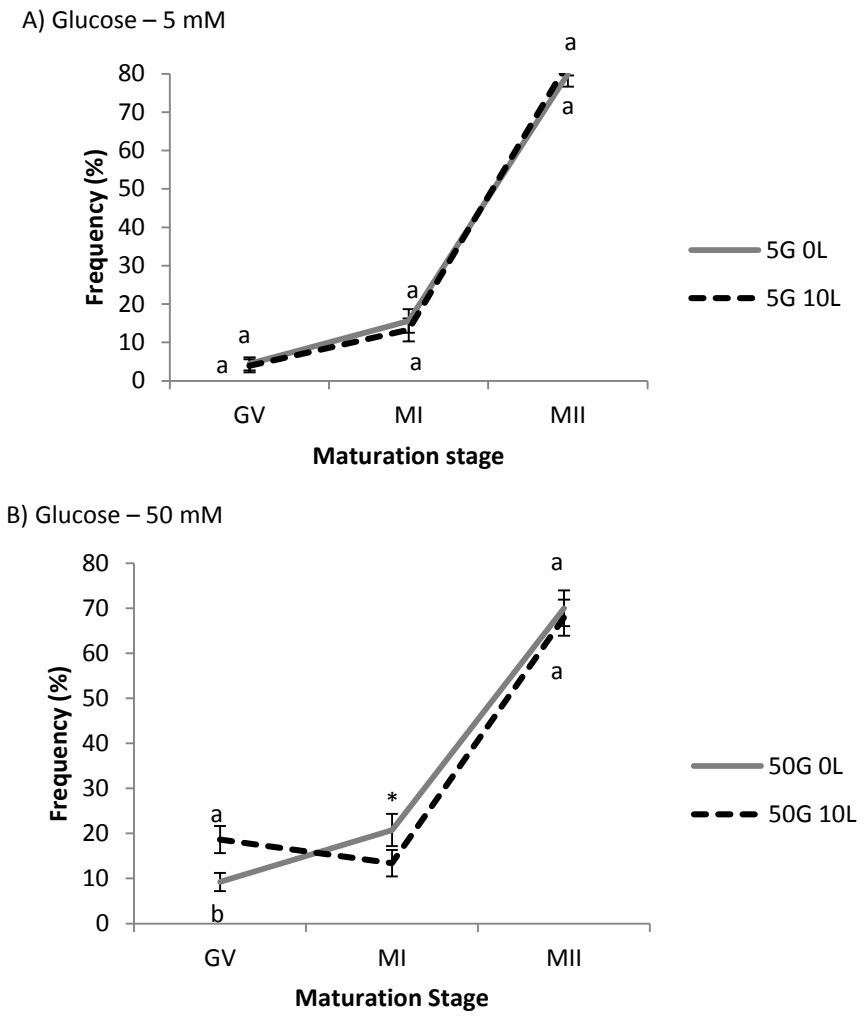

Figure A.2. Effect of physiological concentrations of leptin on porcine oocyte maturation at low (5mM) or high (50 mM) glucose concentrations. A) Oocytes matured in low glucose in the absence of leptin (5G 0L) had similar oocyte maturation compared to oocytes matured in high glucose and 10 mM leptin (5G 10L). B) In the presence of high glucose concentration, leptin (10 L) increased the number of oocytes arrested in GV stage compared to oocytes matured in the absence of leptin (0L). Different superscripts (a,b) differ or tended to differ (*).

Appendix B. Leptin alters androstenedione production by theca cells

The results highlighted in Specific Aim 1 (Silva et al. 2012) are novel in that they defined conclusively that leptin influences on the oocyte depend on glucose concentration. For the first time, it has been demonstrated that oocyte metabolism varies depending on glucose and leptin conditions. Based on this finding, we expect that further analysis of theca cells will allow better characterization of the mechanisms by which the adipokine leptin and glucose act to alter follicular function and oocyte competence.

Results

Presence of leptin receptor in porcine follicles

Leptin receptor is present in porcine follicles collected from abattoir samples. Large porcine follicles (7-9) mm were dissected from abattoir pig ovaries and fixed in ***Dietrich's*** solution. Immunohistochemical staining was performed using the avidin–biotin–peroxidase complex (ABC) method. Sections were treated with a primary rabbit antibody against the short and long form of the leptin receptor (sc-1834-R, Santa Cruz Biotechnology, Santa Cruz, CA). Immunoreactivity was visualized using the chromogen 3,3-diaminobenzidine tetrahydrochloride (DAB) (Santa Cruz Biotechnology, Santa Cruz, CA). Sections were counterstained with hematoxylin (Sigma) solution for 10 sec to visualize nuclei, and mounted on a glass slide. Positive staining is present in granulosa cells, theca cells and ovarian stroma (Figure B.1).

Effect of leptin and insulin on theca cells androstenedione production

Theca cells isolated from small (3-5 mm) and preovulatory (8-12 mm) abattoir-derived porcine follicles were cultured in serum free media and treated with different concentrations of insulin (0 or 100 ng/ml) associated with increasing concentrations of recombinant human leptin (0, 10 and 1000 ng/ml). Media was supplemented with LH (100 ng/ml), FSH (100 ng/ml) and a serum substitute. Experiments were replicated twice, with duplicates cultures per treatment. DNA content in individual wells was determined using PicoGreen fluorometric assay (Invitrogen, Inc.) and steroid accumulation normalized to DNA content. The data were square root transformed for analysis by PROC MIXED in SAS. The use of supra physiological leptin concentrations is suitable in an *in vitro* situation since others have shown that cell culture in the presence of 100 ng/ml of leptin have an intermediate effect on theca and granulosa production, with inhibition only at higher concentrations (Ruiz-Cortes et al. 2003).

There was a significant effect of follicle size, small (3-5 mm) and preovulatory (8-12 mm) follicles, on theca cells production of AND ($P < 0.0001$). Data from small and pre ovulatory follicles were analyzed separately. Cell number, measured by DNA concentration, was positively correlated with androstenedione production by theca cells originated from small follicles. In contrast, the increase on DNA quantity had an inverse correlation with androstenedione production by preovulatory follicles (Figure B.2). During follicle development the number of steroidogenic cells in the theca layer increases until ovulation, when theca and granulosa differentiate to form the corpus luteum. A possible explanation for the non correlation between theca cell number and androstenedione production from preovulatory follicles is the differentiation of these cells to luteal cells (Magoffin D.A., 2005). Future analysis is necessary to determine progesterone concentration and confirm this hypothesis.

Insulin tended to had a positive effect on androstenedione production after 48 hours treatment of theca cells originated from small follicles (Figure B.3); however, exposure to high concentrations of leptin inhibited the positive effect of insulin on androstenedione production of small follicles after 48h of *in vitro* treatment. There was no effect of leptin or insulin on androstenedione production by theca cells originated from pre ovulatory follicles after 24 or 48 hours of treatment (data not shown), demonstrating the different behavior of preovulatory versus small follicles. However, there was an interaction of insulin and leptin which affects DNA content (cell proliferation/survival) of preovulatory follicles 48 h after treatment (Figure B.4) suggesting that these molecules may interfere with cell proliferation. Without leptin, insulin stimulated increased DNA content. However, leptin had a biphasic effect on DNA content when insulin was present. Without insulin, leptin did not affect DNA content.

Further research is necessary to identify the signaling pathway altered by the interaction of leptin and insulin concentrations on theca cells. It is possible that leptin alters gene expression of genes associated with the insulin pathway in theca cells similar to the data illustrated in Specific Aim 1 (Figure B.5). These results verified that oocytes and theca cells function changes based on the interaction between the components present in the *in vitro* system.

FIGURE B.1.

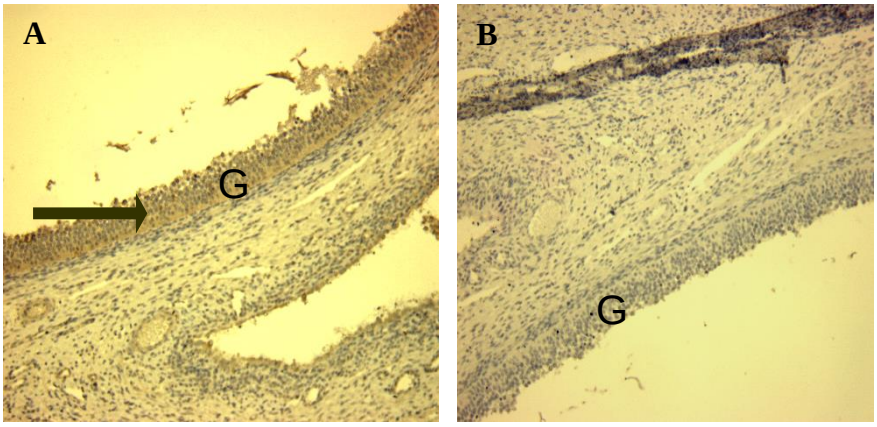

Figure B.1. Detection of leptin receptor in porcine follicles by immunohistochemistry using Ob receptor antibody. A) Leptin receptor indicated by the brown staining (black arrow). Leptin receptor is present in different tissues including granulosa layers of porcine follicles (G). B) Negative control where primary antibody was omitted.

FIGURE B.2.

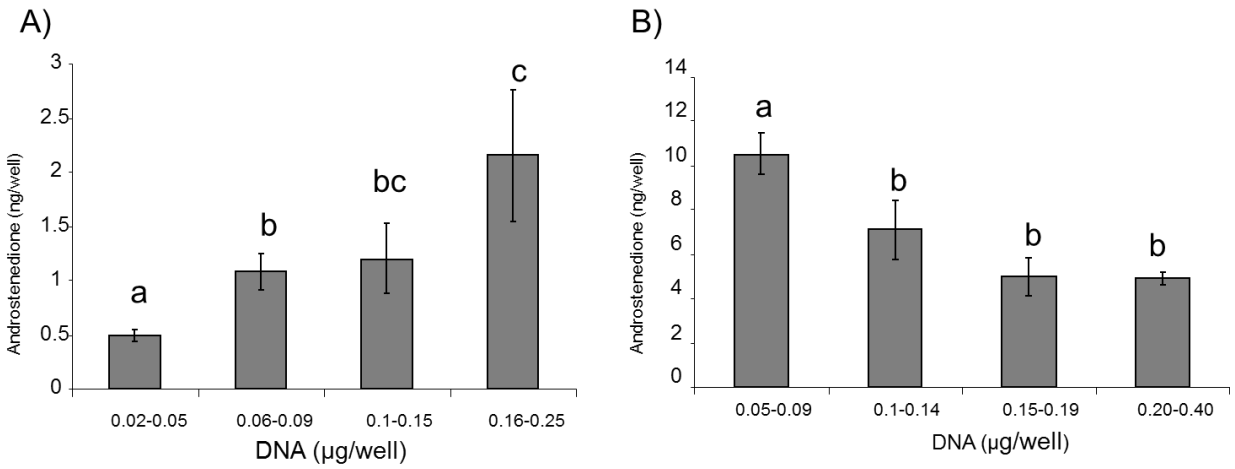

Figure B.2. Effect of follicles size on androstenedione (AND) production. DNA concentration was positively correlated with androstenedione production by theca cells originated from small follicles (A) but had an inverse correlation with androstenedione production by preovulatory follicles (B). Different superscripts (a,b,c) between DNA concentration groups differ (*P* value < 0.05).

FIGURE B.3.

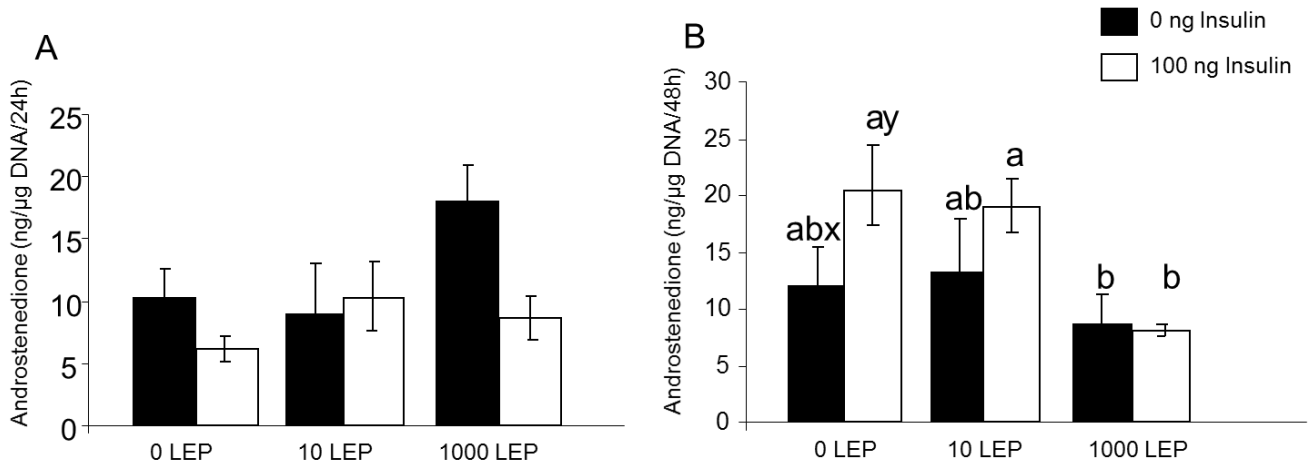

Figure B.3. Effect of treatment on androstenedione (AND) production by porcine small follicles after 24 or 48 hours treatment. A) There was no effect of treatment on androstenedione production after 24 treatment (Treat *P* value > 0.1). B) Insulin and leptin had an effect on androstenedione production 48 h after treatment (Treat *P* value =0.043). Insulin had a tendency to increase androstenedione production by theca cells in the absence of leptin after 48 h of treatment, which is an expected result since insulin is known to increase LH stimulation of theca cells steroidogenesis. Presence of 1000 ng/ml of leptin for 48 hours negated the positive effect of insulin on hormone production with significant decrease in AND production to levels similar to control without insulin. Different superscripts (a,b) between treatments differ. Different superscripts (x,y) between treatments tend to differ (*P* value = 0.07).

FIGURE B.4.

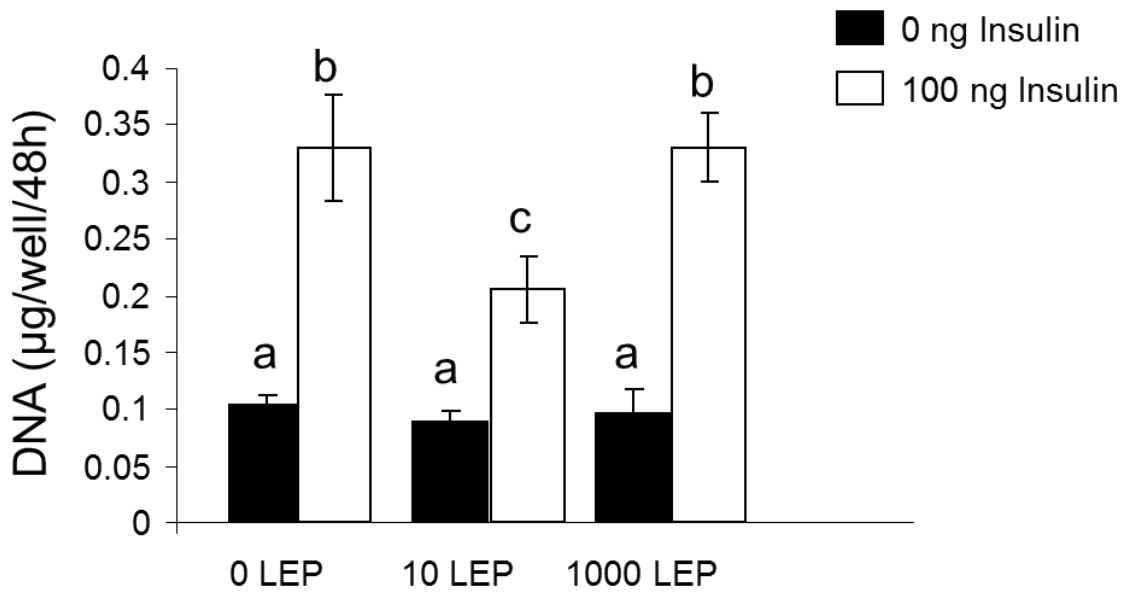

Figure B.4. Effect of leptin (0, 10 or 1000 ng/ml) and insulin concentrations (0 or 100 ng/ml) on theca cells proliferation measured by DNA content at 48 hours after treatment. Theca cells were collected from pre ovulatory follicles.

FIGURE B.5.

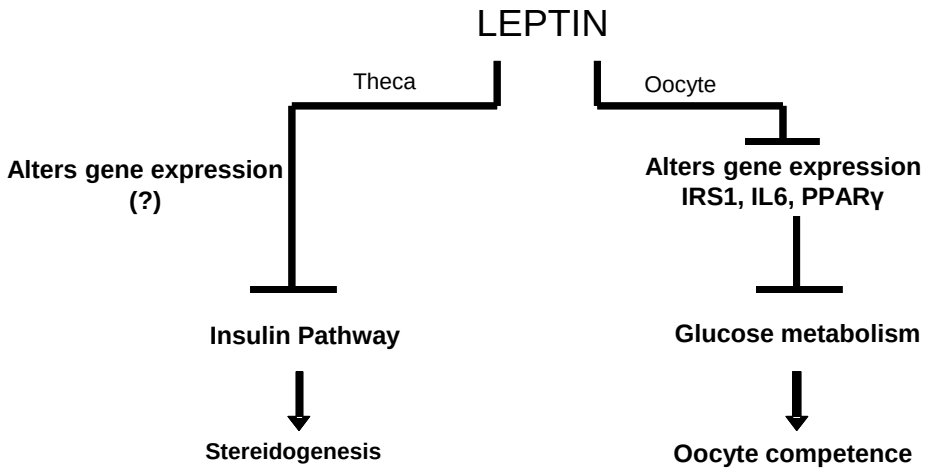

Figure B.5. Proposal model by which leptin alters insulin impact on theca cells production of androstenedione production. Hyperleptinemia may inhibit insulin regulation of theca cells steroidogenesis through an unknown mechanism (Specific Aim 2). Since leptin alters gene expression of genes associated with the insulin pathway in oocytes (Specific Aim 1), it is likely that this mechanism is also present in theca cells.

Appendix C. Importance of lipid metabolism for oocyte nuclear maturation

FIGURE C.1.

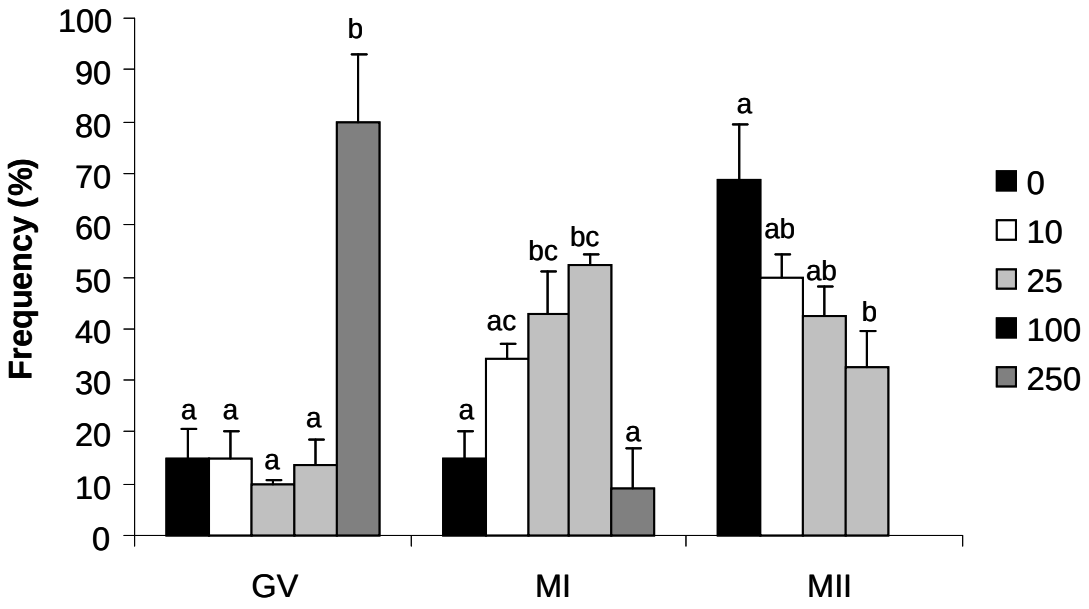

Figure C.1. Maturation status of oocytes exposed to different concentrations of etomoxir (0, 10, 25, 100 and 250 μmoles during *in vitro* maturation. Etomoxir is a fatty acid oxidation inhibitor. Oocytes matured in the presence of 250 μM inhibitor were arrested in GV stage.

GV= germinal vesicle

MI= metaphase I (includes condensed chromatin stage)

MII= metaphase II (includes anaphase and telophase stage)

FIGURE C.2.

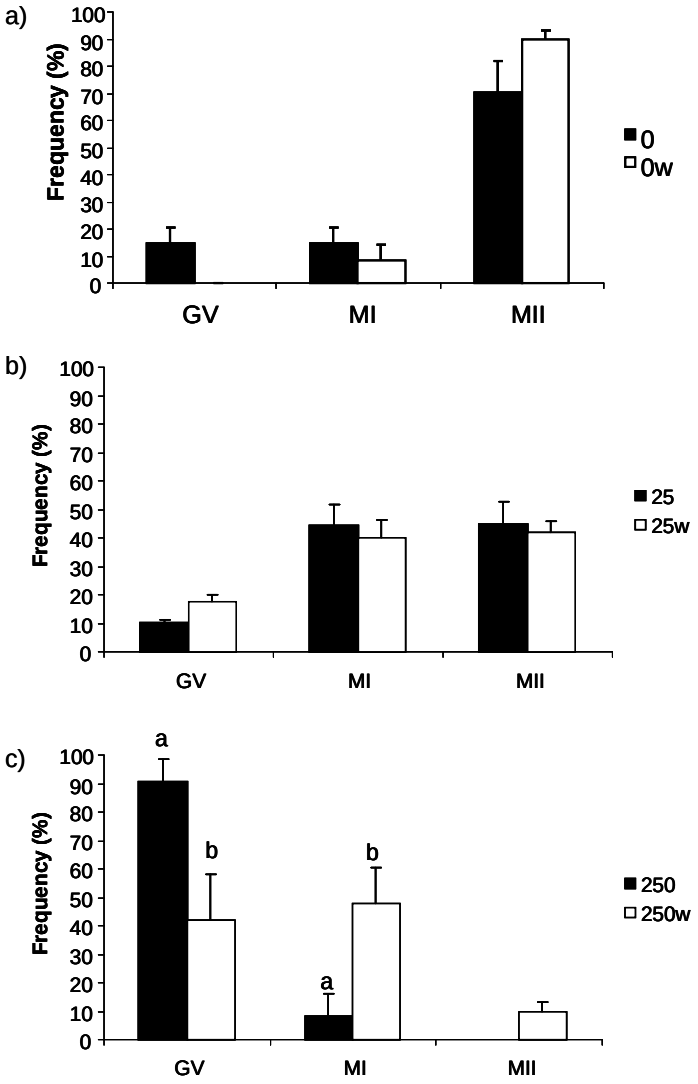

Figure C.2. Determination of inhibitor toxicity on oocytes. Oocytes were matured in the presence of different concentrations of inhibitor (0, 25 or 250 μM) for 24 hours. After the initial period half of the oocytes were moved to a second media without inhibitor (0W, 25W and 250W). Nuclear maturation was determined after 42 h incubation for all groups. Different superscripts (a,b) within a meiotic stage category differ (*P* value < 0.05). In the 250 μM etomoxir treatment group, significantly more oocytes reached MI and MII if inhibitor was present for 24 hours during the maturation period compared to 48 hours demonstrating that oocytes partially recovered from the negative effect of the beta oxidation inhibition.

Figure C.3.

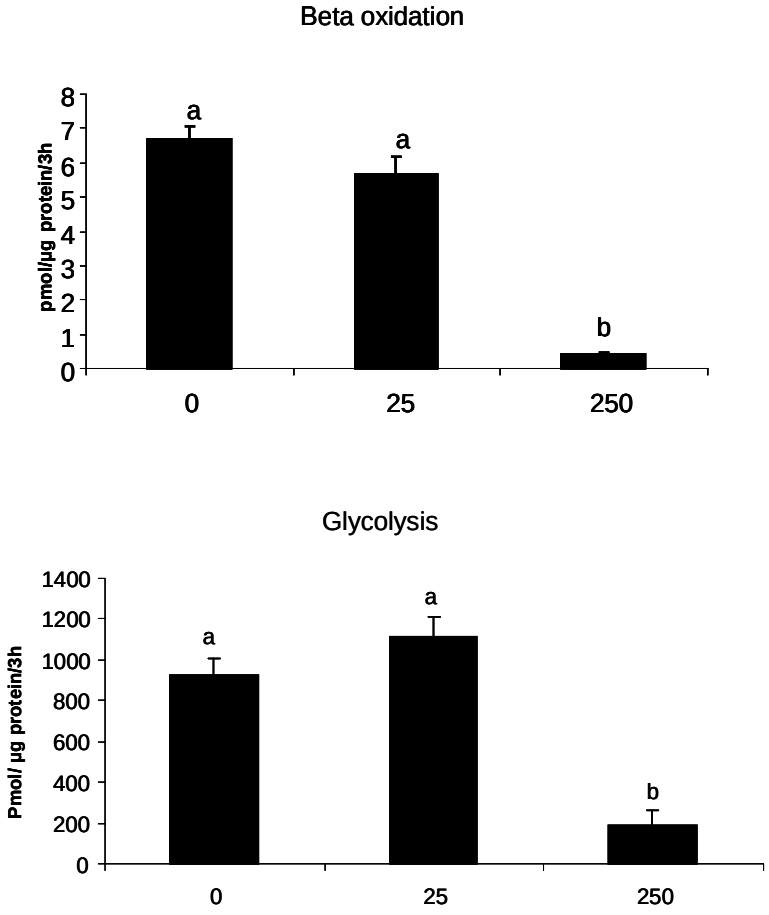

Figure C.3. Alteration of oocytes beta oxidation (top panel) and glycolysis (bottom panel) following treatment with 0, 25 or 250 µM etomoxir. Columns with different letters are significantly different, $P < 0.05$. These results demonstrate that etomoxir at 250 µM concentration significantly inhibit beta oxidation. In addition, glycolysis was also inhibited demonstrated a connection between fatty acid and glucose metabolism.

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